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Multi-targeted metallo-ciprofloxacin derivatives rationally designed and developed to overcome antimicrobial resistance

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ABSTRACT

Antimicrobial resistance is a major global threat to human health due to the rise, spread and persistence of multi-drug-resistant bacteria or 'superbugs'. There is an urgent need to develop novel chemotherapeutics to overcome this overarching challenge. The authors derivatized a clinically used fluoroquinolone antibiotic ciprofloxacin (Cip), and complexed it to a copper phenanthrene framework. This resulted in the development of two novel metallo-antibiotics of general formula $[Cu(N,N)(CipHA)]NO_3$ where N,N represents a phenanthrene ligand and CipHA represents a hydroxamic acid of Cip derivative. Comprehensive studies, including a detailed proteomic study in which *Staphylococcus aureus* cells were exposed to the complexes, were undertaken to gain an insight into their mode of action. These new complexes possess potent antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus*. In addition, they were found to be well tolerated *in vivo* in *Galleria mellonella* larvae, which has both functional and structural similarities to the innate immune system of mammals. These findings suggest that proteins involved in virulence, pathogenesis, and the synthesis of nucleotides and DNA repair mechanisms are most affected. In addition, both complexes affected similar cell pathways when compared with clinically used Cip, including cationic antimicrobial peptide resistance. The Cu-DPPZ-CipHA (DPPZ = dipyrido[3,2-*a*:2',3'-*c*]phenazine) analogue also induces cell leakage, which leads to an altered proteome indicative of reduced virulence and increased stress.

1. INTRODUCTION

Antimicrobial resistance (AMR) is a major health challenge threatening the very core of modern medicine globally [1]. Antibiotics act by targeting different bacterial processes including cell wall and protein synthesis, RNA polymerase and DNA gyrase/topoisomerase action, and membrane structural function [2–4]. These systems are targeted by one or more of four main mechanisms of resistance, namely cell penetration blockage, antibiotic removal by efflux pumps, alteration of the target, and protein inactivation [2,5]. The major antibiotic classes include β-lactams, aminoglycosides, macrolides, tetracyclines, quinolones and glycopeptides, of which quinolones are the third most prescribed [6,7]. The main targets of quinolones and fluoroquinolones are DNA gyrase and topoisomerase enzymes. They act via the formation of a DNA gyrase-quinolone-DNA complex [8] that hinders DNA replication, leading to cellular death in Gram-negative and Gram-positive pathogens [9]. A DNA-binding protein, Qnr protein, which protects quinolone targets from inhibition [10,11], is one of the reasons for low levels of quinolone resistance [12].

Ciprofloxacin (Cip) [Fig. 1(1)] is a second-generation fluoroquinolone that is administered orally or parenterally. It is used as a broad-spectrum antibiotic to treat conditions including respiratory tract infections, urethral infections, skin infections and sinusitis [13]. Structural optimization of Cip has led to derivatives with broad-spectrum activities and minimal toxic side-effects [14].

One of the most effective strategies to overcome AMR development is to generate new structural classes of antibiotics with different chemistries and therefore different mechanisms of action. Metal-based drugs are well suited in this regard. For example, they can exist in variable oxidation states and geometries, and their metal centres can be decorated with therapeutic moieties, including those with antimicrobial properties. The au-

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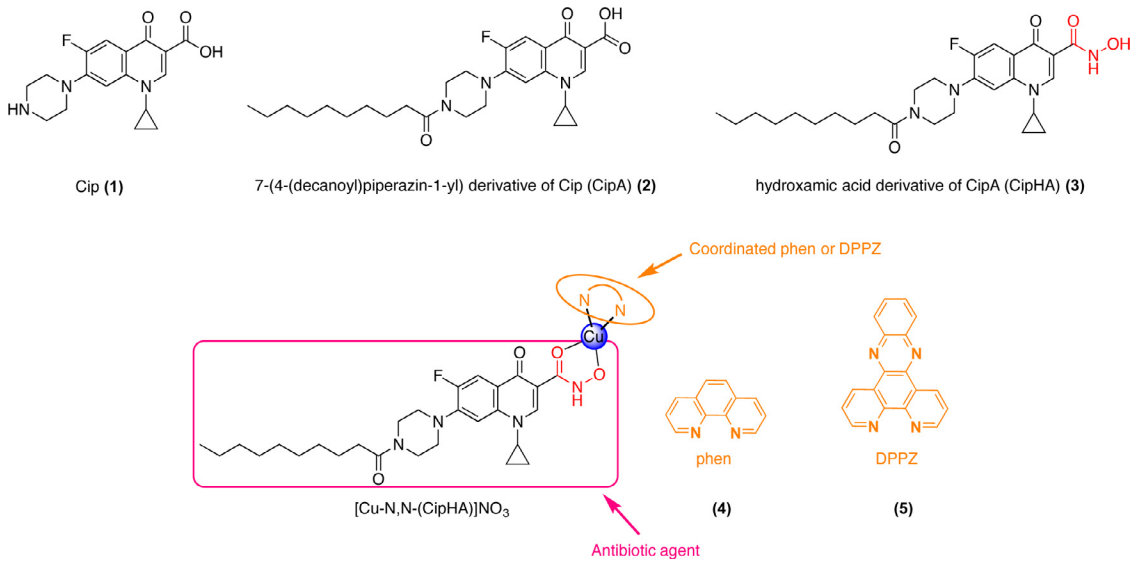

Fig. 1. Chemical structures of ciprofloxacin (Cip) (1), Cip analogue (CipA) (2), Cip analogue hydroxamate (CipHA) (3) and the Cu-N,N-CipHA complexes (4,5).

thors' group recently designed and developed two new classes of metallo-antibiotics [9,15]. The rationale was to develop drugs that combine the antimicrobial properties of Cip while exploiting the properties of either Ru(II) or Cu(II). Complexes containing these metal ions have been widely explored as therapeutic agents [16]. Copper, in particular, has known antimicrobial properties. Although the precise mechanisms behind the antimicrobial activity of copper complexes are not yet fully defined, it appears that the increased production of reactive oxygen species [17] as a result of copper uptake [18] leads to lipid peroxidation [19], loss of membrane integrity and hence cell death [17,18]. Another application of copper is its use as a water disinfectant in a process where copper ions replace silver ions. This action was particularly important for the prevention of spreading of *Legionella* spp., which proved effective in eliminating hospital-acquired Legionnaires' disease in a small-scale study over a 5-year period [20].

The authors recently developed a new class of metallo-antibiotics of general formula [Cu(N,N)(CipA)Cl] where N,N represents a phenanthrene ligand and CipA is a derivative of Cip [Fig. 1(2)] [9]. These novel chemotypes exhibited selective targeting of Gram-positive bacteria, with one lead compound having potency against methicillin-resistant *Staphylococcus aureus* (MRSA) that was an order of magnitude greater compared with the CipA ligand. MRSA is a nosocomial pathogen with high morbidity and mortality [21], and is also widely spread in the community [22].

The authors also recently developed a novel family of Cu(II)-phenanthrene prodrugs incorporating suberoylanilide hydroxamic acid (SAHA), a well-established histone deacetylase inhibitor and clinically approved cancer drug [23]. SAHA was bound to the Cu(II) centre via its hydroxamate *O,O* chelating group. Hydroxamic acids are well known as versatile bioligands and enzyme inhibitors [24]. In an effort to develop new structural classes of existing fluoroquinolone antibiotics, which might lead to different chemistries and different biological properties, the authors decided to derivatize CipA by introducing a hydroxamate metal-binding moiety in place of its carboxylate group, CipHA [Fig. 1(3)]. This was subsequently complexed to a Cu(II)-phenanthrene framework, generating [Cu(N,N)(CipHA)Cl] where N,N is 1,10-phenanthroline/phenanthroline (phen) or dipyrido[3,2-*a*:2',3'-*c*]phenazine (DPPZ) [Fig. 1(4) and (5), respectively].

As the parent metallo-antibiotic [Cu(N,N)(CipA)Cl] family was most active against *S. aureus* and MRSA, attention was focused on the antimicrobial potential of the new [Cu(N,N)(CipHA)Cl] class against these strains, especially given the classification of *S. aureus* as a 'superbug'. Ultimately, the aim was to develop a new class of metallo-antibiotic, rationally designed to overcome AMR. In this article, the rational design and synthesis of two novel complexes of [Cu(N,N)(CipHA)]NO_3, herein referred to as Cu-N,N-CipHA, where N,N stands for phen (4) or DPPZ (5) are reported. Using molecular modelling studies, it was established that incorporation of the hydroxamate moiety did not have an adverse impact on topoisomerase binding, one of the primary targets of Cip. Both Cu-N,N-CipHA complexes and CipHA possessed antimicrobial activities against *S. aureus*, MRSA and, to a lesser extent, Gram-negative bacteria such as *Escherichia coli*. In-vitro studies of amino acid and protein cell wall leakage (see online supplementary material), along with results from the authors' proteomic study, suggest that the complexes mainly affect proteins involved in the synthesis of nucleotides and DNA repair mechanisms, as well as the cationic antimicrobial peptide (CAMP) resistance pathway. In recent years, the use of alternative animal models, such as larvae of the greater wax moth (*Galleria mellonella*), has become increasingly popular given the similarities between the insect immune system and the mammalian innate immune response. *G. mellonella* also represent a more cost-effective in-vivo model with a lack of legal and ethical restrictions [25]. The CipHA and both Cu-N,N-CipHA complexes were well tolerated by the in-vivo model *G. mellonella* larvae (see online supplementary material). These results provide promising evidence that the two Cu-N,N-CipHA complexes and the novel ligand CipHA may be well tolerated *in vivo*.

2. RESULTS AND DISCUSSION

2.1. Syntheses of the Cu-N,N-CipHA complexes and molecular modelling studies

The synthesis of CipA has been reported previously [26] and subsequently optimized [15]. The hydroxamic acid analogue (CipHA) was generated using a protocol reported previously [27] (Fig. 1). Reactions between the Cu(II) precursors, CipHA and phenazine ligands (phen or DPPZ), proved to be robust even when changing the reaction conditions (i.e. presence or absence of base, different solvents used and different order of addition of reagents). The yields and purities of [Cu(phen)(CipHA)]NO_3

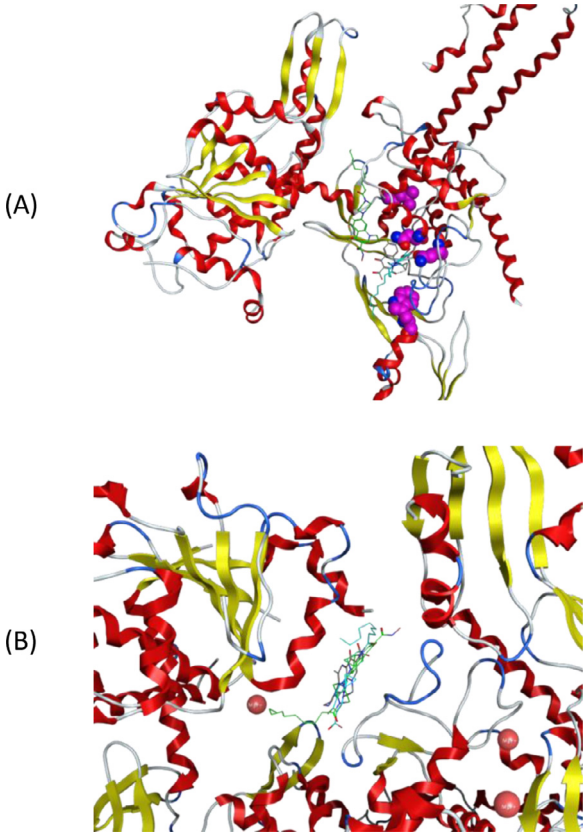

Fig. 2. Summary image of the best docking poses for the three molecules into (A) topoisomerase IIa (protein database (PDB) structure 4FM9 [60]) and (B) topoisomerase IIb (PDB structure 3QX3 [61]). Ciprofloxacin (Cip) has carbons coloured in grey, Cip analogue has carbons coloured in cyan, and Cip analogue hydroxamate has carbons coloured in green.

and [Cu(DPPZ)(CipHA)]NO_3 remained constant. Elemental analyses, mass spectrometry (MS) and infra-red (IR) data all support the formation of the complexes with either phen or DPPZ and CipHA bound to the Cu(II) centres. Despite several attempts, crystals of sufficient quality for X-ray crystallography were not obtained. It is envisaged that in both complexes, the Cu(II) ion binds to the phen or DPPZ ligand via typical *N,N*-bidendate coordination [28]. It is also proposed that the CipHA hydroxamate moiety binds via *O,O*-bidendate coordination, again typical for this class of ligand [29]. For example, Azeredo *et al.* [30] report a crystal structure of a pentacoordinated copper(II) complex incorporating a benzohydroxamate anion, *N*-(2-hydroxybenzyl)-*N*-(2-pyridylmethyl)amine and a chloride anion. Here, the Cu(II) ion is bound to benzohydroxamate via typical *O,O*-bidentate coordination. They provide supporting MS and IR data. The structures proposed for the complexes are also consistent with the authors' literature report for similar Cu(II)-phenanthrene complexes incorporating the hydroxamate-based SAHA [23].

Molecular docking (see S.2 in online supplementary material) was employed to predict the binding affinity of Cip, CipA and CipHA to topoisomerase IIa (Fig. 2A, Table S1, see online supplementary material) and IIb (Fig. 2B, Table S2, see online supplementary material), potential targets for these compounds [31]. Cip, CipA and CipHA all bind to the same region of topoisomerase IIa and topoisomerase IIb. Of the three molecules, the docking score of CipHA demonstrated that it has the strongest predicted affinity for both topoisomerase IIa and IIb, making it a favourable, bidentate ligand for complexation to a Cu-phenazine framework.

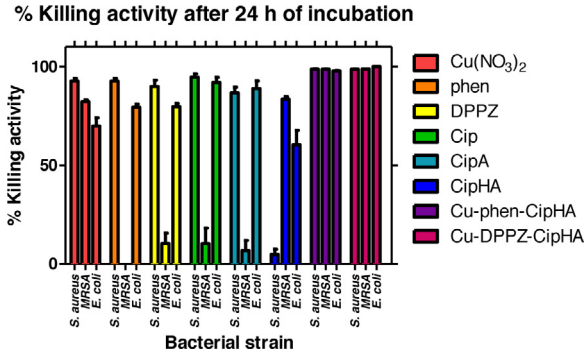

Fig. 3. Percentage killing activity after 24 h of incubation with different agents (100 μg/mL). Data represent mean ± standard error of the mean of the results of three separate determinations. Cip, ciprofloxacin; Phen, 1,10-phenanthroline/phenanthroline; DPPZ, dipyrido[3,2-*a*:2',3'-*c*]phenazine.

2.2. In-vitro antimicrobial activity

Previous reports in the literature suggest that metal-quinolone complexes possess greater antibacterial activity compared with free quinolones [32]. There are specific reports of Cu(II)-Cip complexes where the presence of the copper ion appears to enhance the antimicrobial properties of Cip. For example, Tewes *et al.* [33] demonstrated that the pulmonary delivery of their Cu(II)-Cip complex reduced *Pseudomonas aeruginosa* significantly in a rat chronic lung infection model, while Cip-HCl had no major effect in changing the bacterial lung burden. In the present study, the antimicrobial activities of Cip, CipA, CipHA, phen, DPPZ, $Cu(NO_3)_2$, Cu-phen-CipHA and Cu-DPPZ-CipHA were investigated against selected strains of *E. coli, S. aureus*, MRSA and a yeast (*Candida albicans*) using microbiostatic assays (for all four pathogens) and bactericidal assays (for bacterial pathogens alone) (Table 1, Fig. 3). The assays differ in that the bactericidal assay determines the killing activity of test compounds on plated cells growing on nutrient broth agar, while bacteriostatic assays examine the growth inhibition properties of the test compounds.

Cip showed the greatest bacteriostatic activity with a minimum inhibitory concentration (MIC_{50}) value of approximately 0.98 μg/mL for all three bacterial strains. Surprisingly, CipA did not exhibit any antimicrobial activity with this assay, while CipHA showed bacteriostatic activity with an MIC_{50} value between 31.25 and 62.5 μg/mL in MRSA. After 24 h of incubation, of the Cip derivatives tested, CipHA demonstrated the highest bactericidal activity against MRSA with 83.43 ± 1.29% kill. Cip showed high bactericidal activity after 24 h of incubation against *S. aureus* and *E. coli* with 94.67 ± 1.76% and 91.85 ± 2.67% kill, respectively. Drug-resistant strains often show an altered response to stress and other antimicrobial agents as a result of the changes that occur that make them resistant. These results suggest that MRSA is more sensitive to CipHA than *S. aureus*, possibly as a result of membrane changes associated with the resistant phenotype. Both complexes exhibited good bacteriostatic activity against *S. aureus* and MRSA, with Cu-DPPZ-CipHA being more active, demonstrating activity with MIC_{50} values of approximately 7.8 and 1.95–3.91 μg/mL in *S. aureus* and MRSA, respectively.

Cu-phen-CipHA demonstrated high killing activity after 24 h in all three bacterial strains with kill values >91%. Similarly, Cu-DPPZ-CipHA showed high killing activity after 24 h in *S. aureus* and MRSA, with kill values >93%. The bactericidal activity of Cu-DPPZ-CipHA was shown to be 99.93 ± 0.07% kill in *E. coli*. These promising results in all three bacterial strains for both Cu-phen-CipHA and Cu-DPPZ-CipHA and their lack of in-vivo toxicity towards *G. mellonella* larvae (see online supplementary material) led the au-

Table 1
Minimum inhibitory concentration (MIC_{50}) values (μg/mL) for ciprofloxacin (Cip), Cip analogue (CipA), Cip analogue hydroxamate (CipHA), 1,10-phenanthroline/phenanthroline (phen), dipyrido[3,2-*a*:2',3'-*c*]phenazine (DPPZ), $Cu(NO_3)_2$, Cu-phen-CipHA and Cu-DPPZ-CipHA in four different pathogens. Results were determined in triplicate.

Pathogen Compound	*Staphylococcus aureus*	MRSA	*Escherichia coli*	*Candida albicans*
$Cu(NO_3)_2$	>250	>250	~250	>250
Phen	~7.81–15.63	~15.63	~7.81	~7.81–15.63
DPPZ	>250	>250	>250	~15.63–31.25
Cip	< 0.98	< 0.98	~0.98–1.95	>250
CipA	>250	>250	>250	>250
CipHA	>250	~31.25–62.50	>250	>250
Cu-phen-CipHA	~62.50	~15.63	>250	>250
Cu-DPPZ-CipHA	~7.81	~1.95–3.91	>250	>250

MRSA, methicillin-resistant *Staphylococcus aureus*.

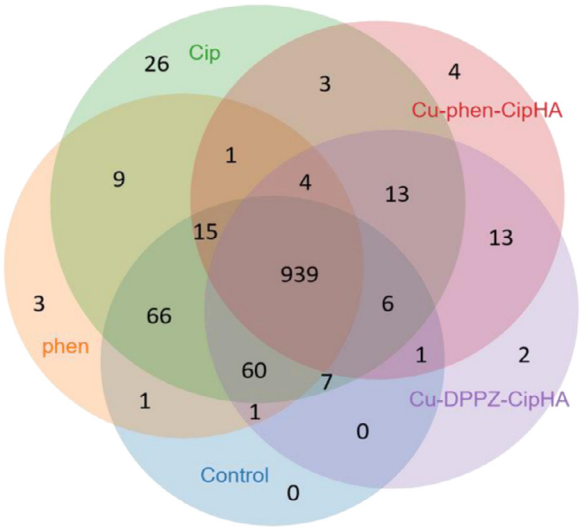

Fig. 4. Venn diagram representing relationship between all statistically significant differentially abundant (SSDA) proteins of five different treatments. Not shown on the diagram are the following SSDA proteins in common: Control/Cip – 21; Cip/Cu-DPPZ-CipHA – 8; phen/Cu-phen-CipHA/Cu-DPPZ-CipHA – 2; Control/Cip/Cu-phen-CipHA – 1 and phen/Cip/Cu-DPPZ-CipHA – 3. Cip, ciprofloxacin; CipHA, Cip analogue hydroxamate; DPPZ, dipyrido[3,2-*a*:2',3'-*c*]phenazine; Phen, 1,10-phenanthroline/phenanthroline.

thors to conduct a full proteomic investigation into *S. aureus* cells to gain a deeper insight into their protein targets and hence modes of action.

2.3. Proteomic analysis of S. aureus exposed to novel Cu-N,N-CipHA complexes

2.3.1. Protein identification and quantification

High-resolution quantitative MS can identify and quantify thousands of proteins in a single run, which gives an unprecedented opportunity to examine changes in the proteomic profile of an organism (e.g. *S. aureus*) exposed to stress or another condition. Label-free quantitative proteomic analysis was conducted on *S. aureus* cells (5×10^8/mL) exposed to phen, Cip, Cu-phen-CipHA and Cu-DPPZ-CipHA for 5 h. In total, 22,470 peptides were identified, representing 1209 proteins with two or more peptides. After 24 h, a total of 82 [phen vs. control (5 mL of dimethyl sulfoxide; final concentration 10%)], 172 (Cip vs. control), 461 (Cu-phen-CipHA vs. control) and 278 (Cu-DPPZ-CipHA vs. control) proteins were determined to be differentially abundant (analysis of variance, $P<0.05$) with a fold change >1.5 (Fig. 4).

In total, three (phen vs. control), 26 (Cip vs. control), four (Cu-phen-CipHA vs. control) and two (Cu-DPPZ-CipHA vs. control) proteins were deemed exclusive (i.e. with label free identification (LFQ) intensities present in all three replicates of one treatment, and absent in all three replicates of the other four treatments). These proteins were also used in statistical analysis of the total differentially expressed group following imputation of the zero values as described. A principal component analysis performed on all filtered proteins distinguished the control, phen-, Cip-, Cu-phen-CipHA- and Cu-DPPZ-CipHA-treated samples, indicating a clear difference between each proteome (Fig. S4, see online supplementary material).

Volcano plots for all the treatments were generated to determine which proteins are increased or decreased in abundance after exposure of *S. aureus* to test agents (Fig. 5).

The biggest effect was observed in the case of Cu-phen-CipHA vs. control (461 proteins, 15.95% of proteome changed in abundance) and in the case of Cu-DPPZ-CipHA vs. control (278 proteins, 9.62%) compared with the proteome of *S. aureus* (2889 total proteins). In the case when *S. aureus* cells were exposed to phen for 5 h (Table S3, see online supplementary material), and if compared with the control, the proteins which increased in relative abundance were *S. aureus* surface proteins 1 and J (19 and 11.2 fold), three iron-regulated surface determinant proteins (from 19 fold to 11.2 fold), copper chaperone CopZ (11.4 fold), copper-exporting P-type ATPase (9.1 fold), IgG binding protein A (3 fold) and alcohol dehydrogenase (1.6 fold). The proteins which decreased in relative abundance were penicillin binding protein 3 (1.6 fold), penicillin binding protein 2 (2.3 fold), lipoteichoic acid synthase (2.3 fold) and staphopain B (2.5 fold).

After exposing *S. aureus* cells to Cip for 5 h (Table S4, see online supplementary material), the proteins which increased in relative abundance were nuclease SbcCD subunit C (45.5 fold), proteins of the exonuclease family, fibronectin binding protein (3.7 fold), iron ABC transporters (with 3.7 fold the highest), DNA topoisomerase IV subunit A (2.4 fold) and subunit B (2.2 fold), DNA polymerase (1.9 fold) and a range of ribosomal proteins (from 1.5 fold to 2.3 fold). The proteins that decreased in relative abundance were penicillin binding protein 3 (1.6 fold), penicillin binding protein 2 (2.3 fold) and lipoteichoic acid synthase (2.3 fold).

When *S. aureus* was exposed to Cu-phen-CipHA for 5 h (Table S5, see online supplementary material), the following proteins were increased in abundance: probable autolysin SsaALP (112 fold); UPF0291 protein (87 fold); secretory antigen SsaA, lipase 1, delta-hemolysin and coagulase (all from 35 fold to 54 fold); SOD (5.4 fold); staphylococcal complement inhibitor (3 fold); a range of ribosomal proteins; RNA polymerase subunits; DNA binding proteins; proteins involved in protein synthesis; and proteins involved in glycolysis. Also, several proteins decreased in abundance, namely immunodominant staphylococcal antigen B (22.6 fold), penicillin binding protein 2 (5.5 fold), staphylococcal protein A (4.8 fold), staphylococcal secretary antigen ssA2 (4.4 fold), DNA repair pro-

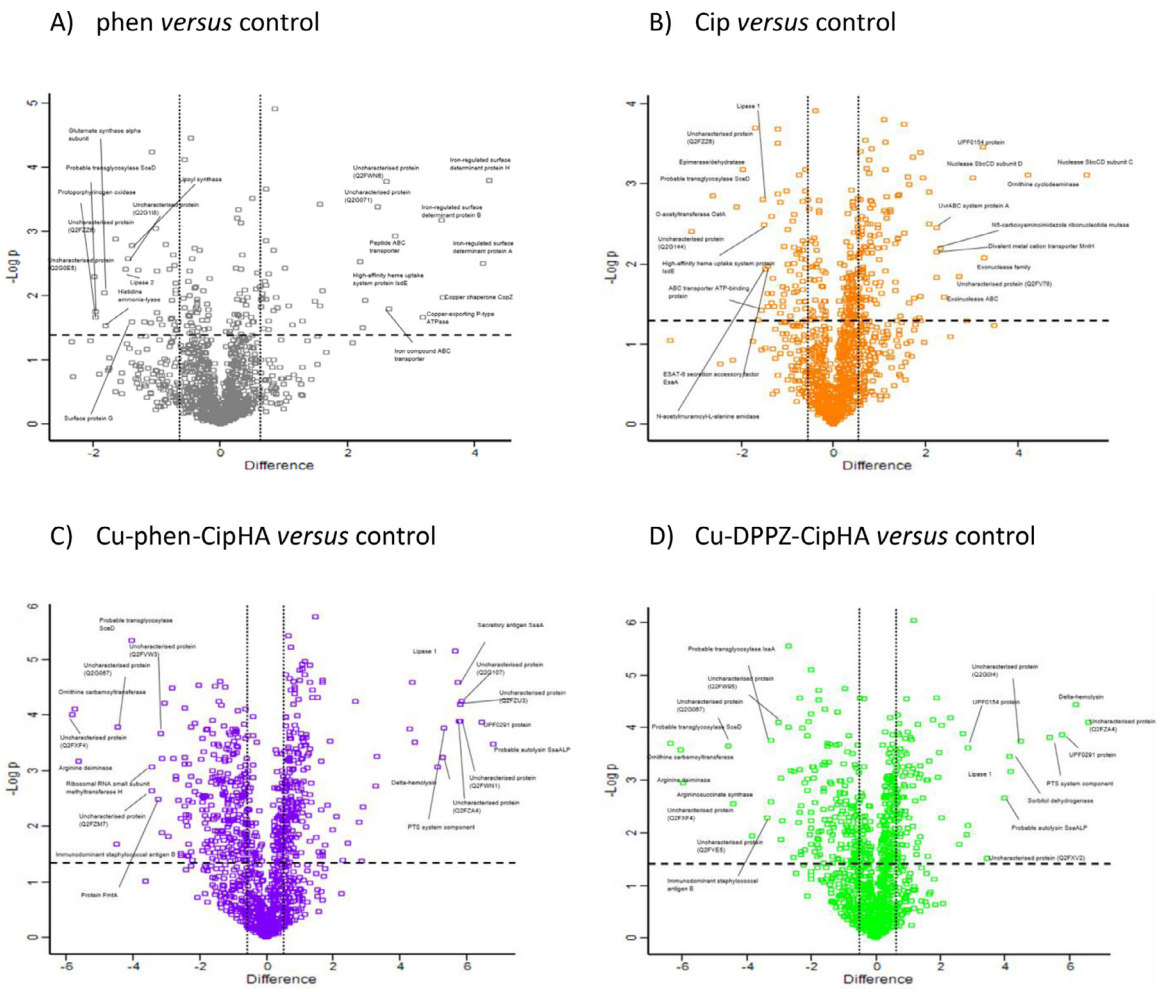

Fig. 5. Volcano plots showing which proteins are increased/decreased in abundance after different treatments. Cip, ciprofloxacin; CipHA, Cip analogue hydroxamate; DPPZ, dipyrido[3,2-*a*:2',3'-*c*]phenazine; Phen, 1,10-phenanthroline/phenanthroline.

Table 2
Top five proteins that changed in abundance when *Staphylococcus aureus* was exposed to the Cu-N,N-CipHA complexes relative to ciprofloxacin (Cip).

	Cu-phen-CipHA	Cu-DPPZ-CipHA
Increased compared with Cip	UPF0291 protein SAOUHSC_02893	PTS system component, putative
	Lipase 1 (EC 3.1.1.3) (glycerol ester hydrolase 1)	UPF0291 protein SAOUHSC_02893
	Secretory antigen SsaA, putative	Lipase 1 (EC 3.1.1.3) (glycerol ester hydrolase 1)
	PTS system component, putative	Delta-hemolysin (delta-lysin) (delta-toxin)
	Probable autolysin SsaALP (EC 3.5.1.28)	Cold shock protein, putative
Decreased compared with Cip	Arginine deiminase (EC 3.5.3.6) (arginine dihydrolase)	Arginine deiminase (EC 3.5.3.6) (arginine dihydrolase)
	Ornithine carbamoyltransferase (EC 2.1.3.3)	Ornithine carbamoyltransferase (EC 2.1.3.3)
	Nuclease SbcCD subunit C	Nuclease SbcCD subunit C
	N5-carboxyaminoimidazole ribonucleotide synthase (N5-CAIR synthase) (EC 6.3.4.18) (5-(carboxyamino)imidazole ribonucleotide synthetase)	Probable transglycosylase SceD (EC 3.2.-.-)
	Ribosomal RNA small subunit methyltransferase H (EC 2.1.1.199) (16S rRNA m(4)C1402 methyltransferase) (rRNA (cytosine-N(4)-)-methyltransferase RsmH)	Probable transglycosylase IsaA (EC 3.2.-.-) (immunodominant staphylococcal antigen A)

CipHA, Cip analogue hydroxamate; DPPZ, dipyrido[3,2-*a*:2',3'-*c*]phenazine; Phen, 1,10-phenanthroline/phenanthroline.

teins, purine metabolism proteins and several penicillin binding proteins (from 2.6 fold to 5.5 fold). The second complex of the series, Cu-DPPZ-CipHA (Table S6, see online supplementary material), affected the increase in abundance of conserved virulence factor B (1.6 fold), SOD (7 fold), alcohol dehydrogenase (6.5 fold), catalyse (1. 5 fold), thioredoxin (1.6 fold), a range of ribosomal proteins (1.5 fold to 3.2 fold), delta-hemolysin (74 fold), coagulase (7.3 fold), gamma-hemolysin (4.1 fold), gamma-hemolysin component C (2.8 fold), alpha-hemolysin (2.6 fold), a range of proteins from the exonuclease family (6.1 fold), transcription regulator CtsR (3.1 fold), transcription regulator SarR (3 fold), helix-turn-helix domain protein (2.4 fold), transcription regulator MgrA (2.1 fold) and purine nucleoside phosphorylase (2.1 fold). The same complex affected a decrease in abundance of several penicillin binding proteins (from 1.8 fold to 4.2 fold) and immunodominant staphylococcal antigen B (10 fold).

To investigate if there was any advantage in using Cu-DPPZ-CipHA over Cu-phen-CipHA (Table S7, see online supplementary

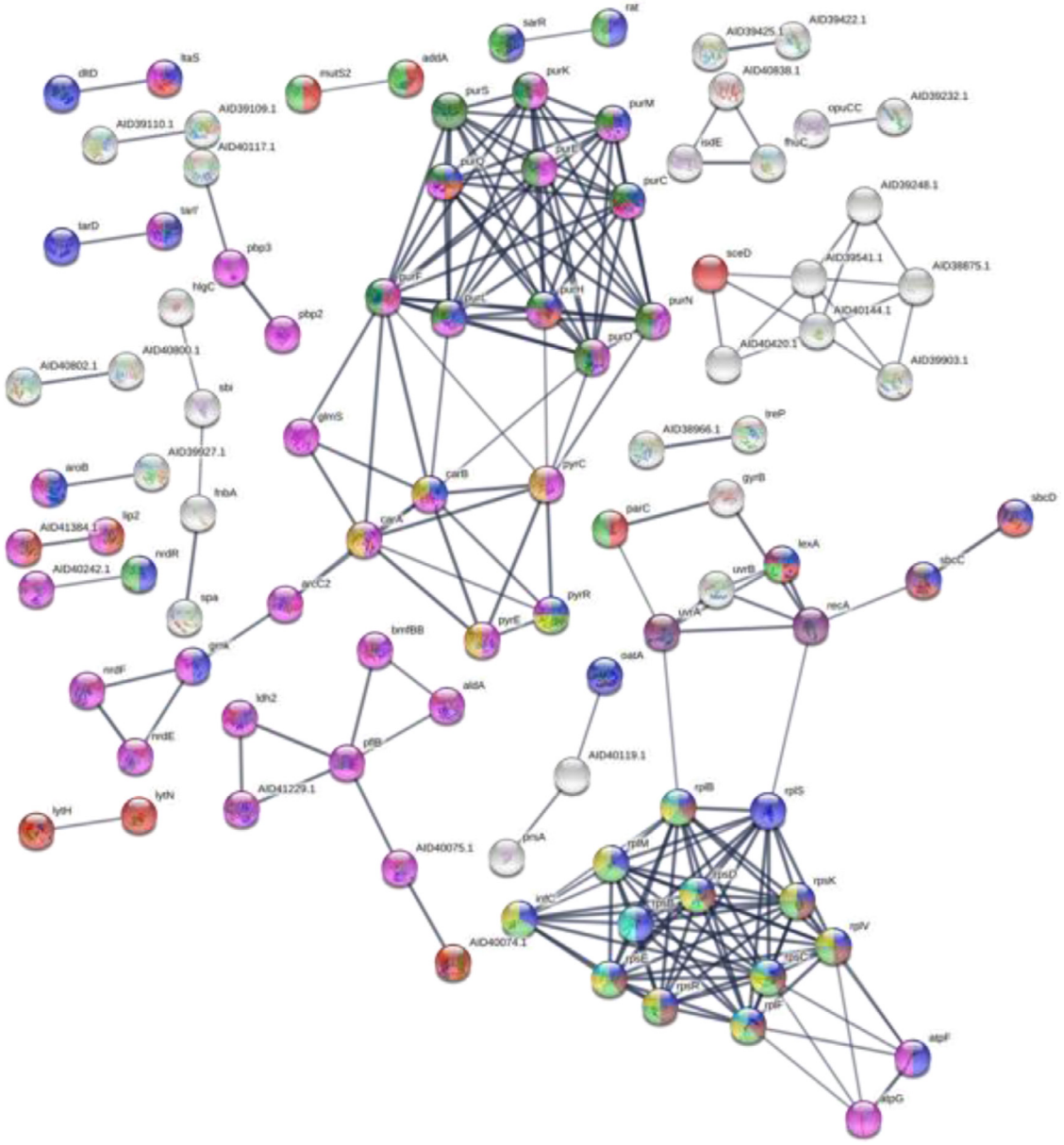

Fig. 6. STRING results for each of the treatments representing the most affected processes, functions and part of cells. (A) Ciprofloxacin (Cip) vs. control; blue, cellular biosynthetic process; brown, rRNA binding; red, hydrolase activity; green, nucleic acid binding; yellow, RNA binding; purple, metabolic pathways; turquoise, ribosome; dark green, purine biosynthesis; dark purple, SOS response; ochre, pyrimidine biosynthesis. (B) 1,10-phenanthroline/phenanthroline (phen) vs. control; blue, metal binding; red, extracellular region; green, cell wall; yellow, ion binding; purple, iron; turquoise, periplasmic binding protein; ochre, iron transport-associated domain. (C) Cu-phen-CipHA vs. control; blue, cellular process; brown, metabolic pathways; red, metabolic process; green, biosynthesis of antibiotics; yellow, intracellular part; purple, catalytic activity; turquoise, biosynthesis of secondary metabolites; dark green, binding; dark purple, signal; ochre, cellular biosynthetic process. (D) Cu-DPPZ-CipHA vs. control; blue, metabolic process; brown, pathogenesis; red, cellular process; green, secreted; yellow, protein metabolic process; purple, cellular metabolic process; turquoise, cell part; dark green, peptide metabolic process; dark purple, signal; ochre, virulence; circle, β-lactam antibiotic resistance. CipHA, Cip analogue hydroxamate; DPPZ, dipyrido[3,2-*a*:2',3'-*c*]phenazine.

material), the results for both complexes were compared. It was established that several proteins were affected to a greater extent by Cu-DPPZ-CipHA. More specifically, there was increased abundance of proteins such as ribosomal RNA small subunit methyltransferase H (7.9 fold), glutamate racemase (5 fold), N5-carboxyaminoimidazole ribonucleotide synthase (4.9 fold) and ATP-dependent DNA helicase PcrA (4.5 fold). These results suggest that Cu-DPPZ-CipHA mainly affects cell wall biosynthesis, metal ion binding and DNA replication [34–36]. In addition, numerous proteins were also decreased in abundance after incubation with Cu-DPPZ-CipHA compared with incubation with Cu-phen-CipHA, such as secretory antigen SsaA (30.5 fold), thermonuclease (9.2 fold) and fibronectin binding protein B (9 fold), with pathogenesis, metal ion binding and the stress response affected to the greatest extent [36–38].

Next, the levels of protein abundance were compared when *S. aureus* was exposed to both Cu-N,N-CipHA complexes relative to Cip. The top five proteins that either increased or decreased in abundance for both complexes relative to Cip are shown in Table 2 (Tables S9 and S10, see online supplementary material).

The results suggest that both Cu-N,N-CipHA complexes affect similar processes in *S. aureus*, such as catabolic processes, sugar transport systems, cleavage of DNA hairpin structures and arginine degradation [36,39–42]. These processes did not seem to be affected when treated with Cip alone, thus differentiating these Cu-N,N-CipHA complexes from the clinically used Cip. In addition,

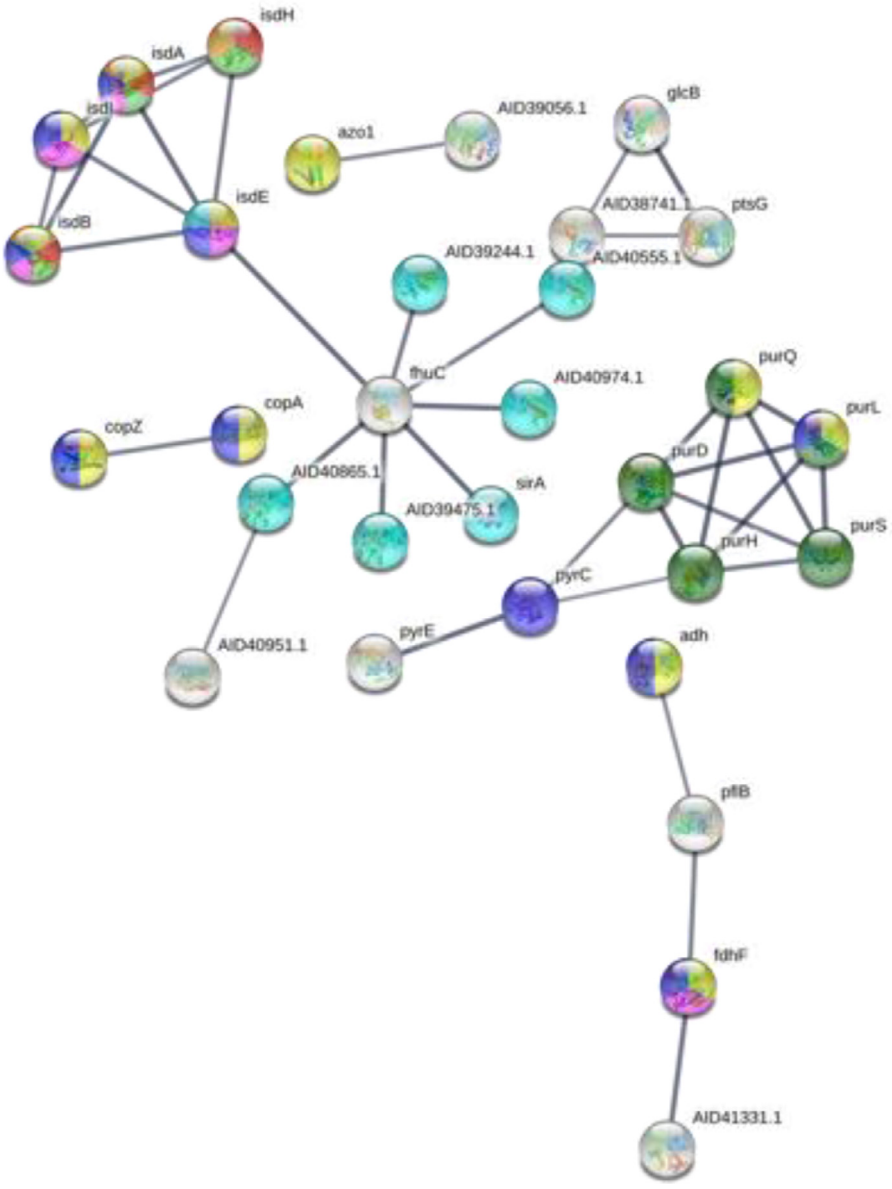

Fig. 6. Continued

Cu-phen-CipHA also influences biosynthesis of amino acids, degradation of cell wall, binding of metal ions and processing of rRNA [34,36,38,43]. Interestingly, Cu-DPPZ-CipHA showed a greater effect on proteins involved in shock and virulence [36,44,45]. The latter complex also affects proteins that are necessary for normal growth under stressful conditions [36].

2.3.2. Functional classification of differently expressed proteins

Blast2GO was used to identify several gene ontology (GO) terms altered by each treatment (Fig. S5, see online supplementary material). For phen, several biological processes (BP) (interspecies interaction between organisms), molecular functions (MF) (cofactor binding, transmembrane transporter activity) and cellular components (CC) (periplasmic space, envelope and external encapsulating structure) were enriched. Treatment of *S. aureus* with Cip enriched the following GO terms involved in BP (cellular component organization, cellular response to stimulus) and in CC (intracellular organelle, non-membrane-bound organelle, ribonucleoprotein complex, intracellular organelle part). Of both complexes, Cu-DPPZ-CipHA caused a greater change in upregulation of processes and functions. This complex specifically caused upregulation in interspecies interaction between organisms, cellular component organization, cellular response to stimulus, regulation of cellular process, response to stress and catalytic activity. The Cu-phen-CipHA complex was involved with the upregulation of cellular processes and cofactor binding. It has to be noted here that the BP, MF and CC impacted by each complex are a combination of the BP, MF and CC for Cip and phen alone. Given that DPPZ is structurally similar to phen, it was expected that the Cu-DPPZ-CipHA complex would behave in a similar fashion to the phen complex.

To further investigate proteomic changes in *S. aureus* exposed to the test agents (phen, Cip, Cu-phen-CipHA and Cu-DPPZ-CipHA), STRING analysis was employed (Fig. 6). Cip [Fig. 6(A)] affected several processes in *S. aureus*, namely cellular biosynthetic processes, rRNA binding, hydrolase activity, nucleic acid binding, RNA binding, purine biosynthesis, pyrimidine biosynthesis, several metabolic pathways and SOS response. The cellular component most affected was the ribosome. Phen [Fig. 6(B)] had a lesser effect on *S. aureus* cells compared with Cip, mainly affecting metal binding and ion binding proteins as well as iron transport and the periplasmic

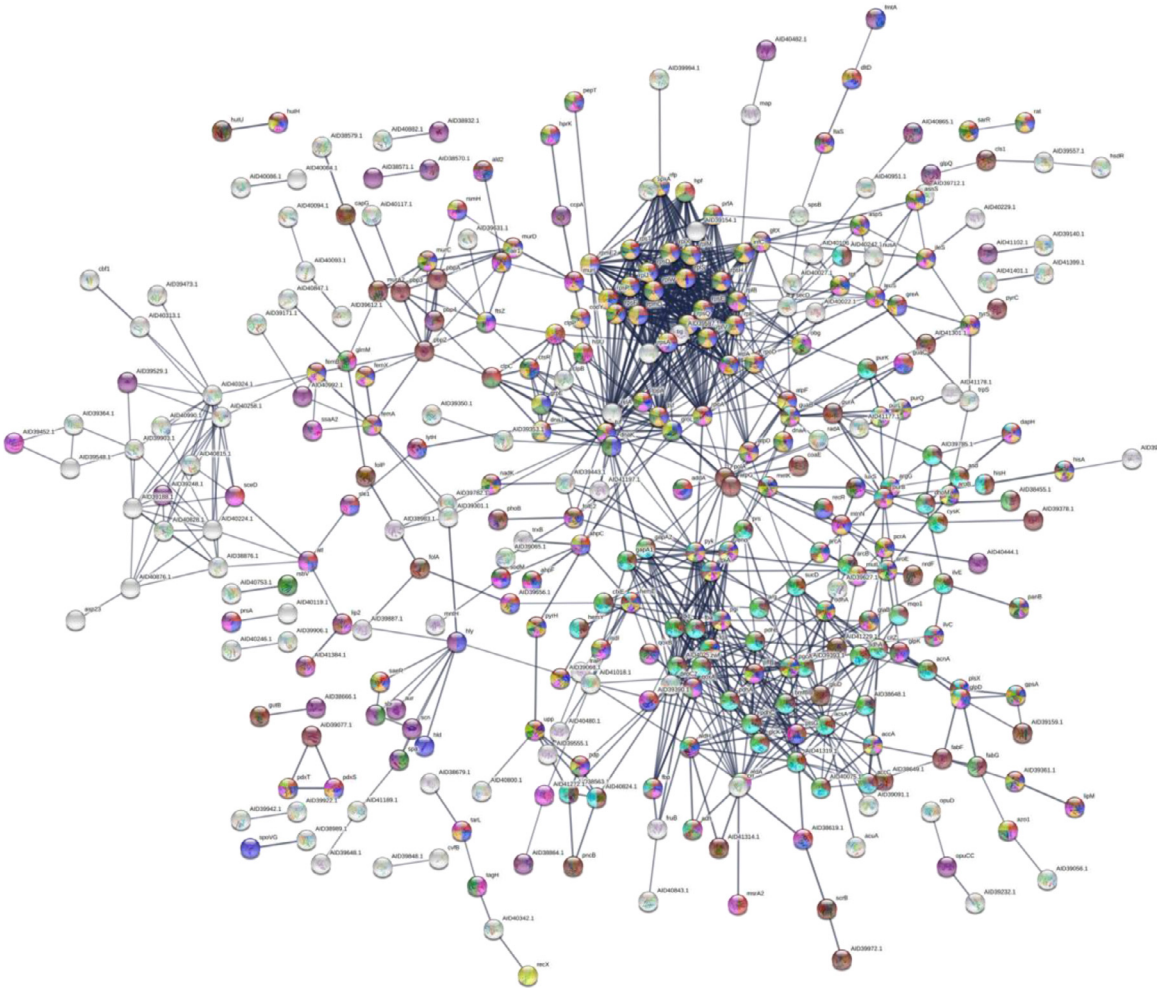

Fig. 6. Continued

binding protein associated domain. Moreover, phen affected several cellular components of *S. aureus*, with the extracellular region and cell wall being the most influenced. STRING analysis proved to be particularly useful when analysing the data for both complexes. Exposing *S. aureus* cells to Cu-phen-CipHA [Fig. 6(C)] resulted in several altered cellular processes (e.g. metabolic pathways, metabolic processes, binding, biosynthesis of antibiotics and biosynthesis of secondary metabolites, cellular biosynthetic processes and catalytic activity). The most altered cellular component was found to be the intracellular part. Similar cell processes in *S. aureus* were affected when exposed to Cu-DPPZ-CipHA. Of note, amongst the numerous altered processes, proteins involved in signalling, virulence, pathogenesis and β-lactam antibiotic resistance were most affected (marked with a circle) [Fig. 6(D)].

Automatic annotation and KEGG mapping service were also used to determine potential cell pathways affected by the complexes, shown in the online supplementary material (S.7).

3. CONCLUSIONS

Two novel Cu(II)-Cip chemotypes of general formula [Cu(N,N)(CipHA)]NO_3 have been synthesized where N,N represents phen or DPPZ and CipHA, a hydroxamic acid derivative of the clinically used fluoroquinolone antibiotic Cip. CipHA showed antibacterial activity towards MRSA and not towards *S. aureus*. Both novel complexes exhibited significant antibacterial activity towards MRSA and were also very potent against *S. aureus*. The Cu-DPPZ-CipHA complex, in particular, was found to be more active than Cu-phen-CipHA against MRSA. When *S. aureus* cells were incubated with Cu-DPPZ-CipHA, proteins and amino acid leakage were observed, suggesting a role for this family of complexes in enhancing permeability of cells. In addition, it was established that CipHA and both complexes were well tolerated by a widely adopted in-vivo insect model, which is a positive predictor for their future development. Comprehensive proteomic studies in which *S. aureus* cells were exposed to the Cu-N,N-CipHA complexes were undertaken to gain an insight into their potential protein targets. These findings suggest that proteins involved in virulence, pathogenesis and the synthesis of nucleotides and DNA repair mechanisms are most affected. In addition, both complexes exhibited an effect on proteins involved in DNA repair, with Cu-phen-CipHA downregulating and Cu-DPPZ-CipHA upregulating these proteins. Conversely, both complexes upregulated proteins associated with virulence, the stress response and pathogenesis. Both complexes also retained a similar effect on proteins associated with DNA synthesis, repair and binding when compared with Cip. Of significance is the fact that both complexes also exhibited effects on CAMP resistance similar to Cip [46,47]. Moreover, Cu-phen-CipHA affected the process of breaking cell walls, and Cu-DPPZ-CipHA was shown to be involved with proteins associated with multi-drug resistance. These results are in accordance with literature reports of the proteome of pathogens undergoing stress due to treatments with antimicrobials [34–36,38,43,48–59]. Moreover, based on the aforementioned STRING analysis,

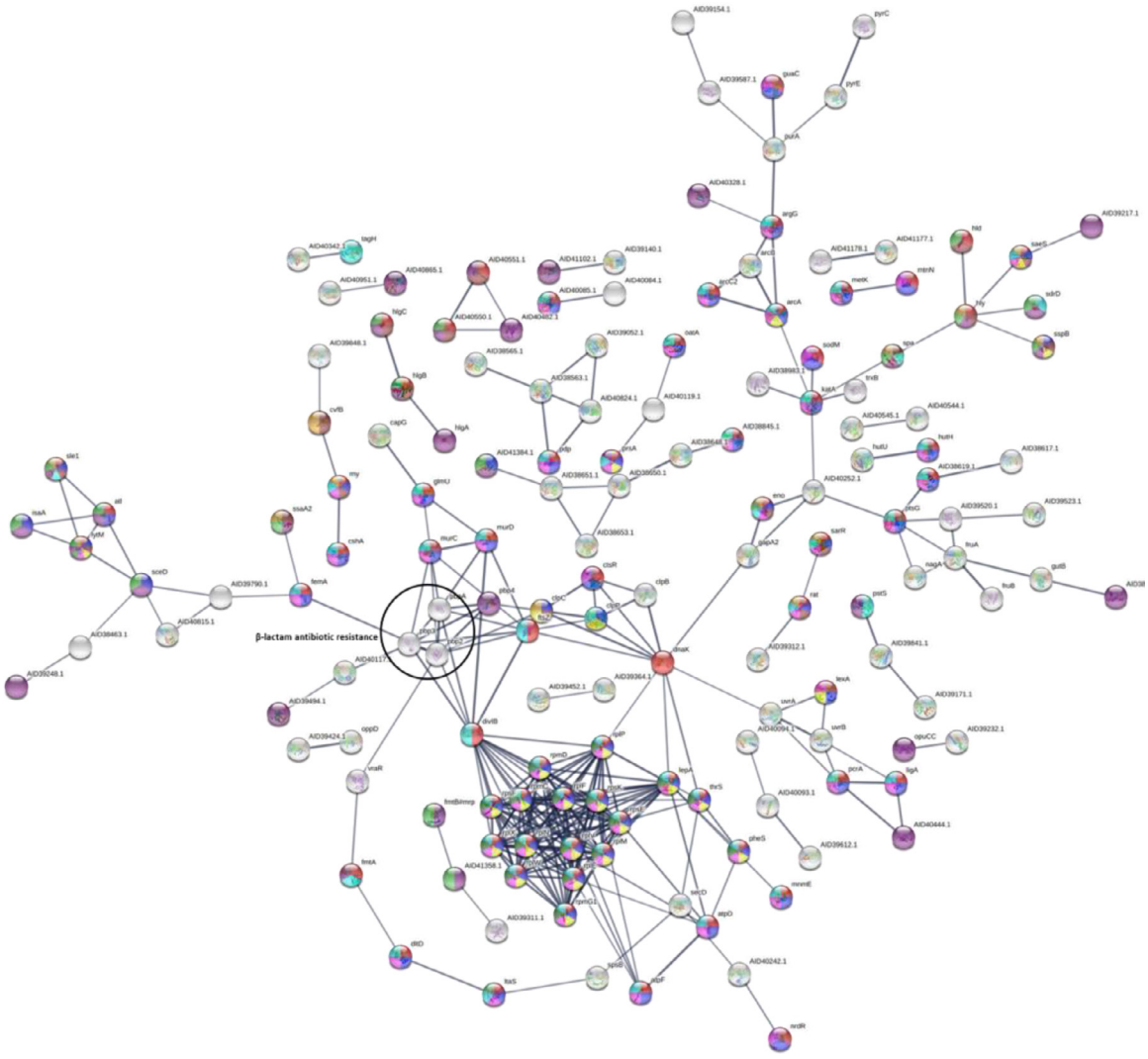

Fig. 6. Continued

Cu-DPPZ-CipHA also showed an effect on β-lactam antibiotic resistance, making this complex a very interesting target for further development. The selected lead candidate, Cu-DPPZ-CipHA, is responsible for inhibition of cell growth and killing of cells, and it induces cell leakage (implying osmotic and/or oxidative stress) which leads to altered proteome indicative of reduced virulence and increased stress. Given that antibiotic resistance in *S. aureus* has become a major global challenge, there is an urgent need to identify new approaches to cater for the anticipated further evolution of resistance. Taking all these findings into consideration, these complexes, but particularly Cu-DPPZ-CipHA as the lead drug candidate, have significant promise to act as metallo-antibiotics in their own right.

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Competing interests

None declared.

Ethical approval

Not required.

Author contributions

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2021.106449.

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