Antimicrobial and Antibiofilm Activities of Helical Antimicrobial Peptide Sequences Incorporating Metal-Binding Motifs

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S *Supporting Information*

ABSTRACT: Antimicrobial peptides (AMPs) represent alternative strategies to combat the global health problem of antibiotic resistance. However, naturally occurring AMPs are generally not sufficiently active for use as antibiotics. Optimized synthetic versions incorporating additional design principles are needed. Here, we engineered amino-terminal Cu(II) and Ni(II) (ATCUN) binding motifs, which can enhance biological function, into the native sequence of two AMPs, CM15 and citropin1.1. The incorporation of metal-binding motifs modulated the antimicrobial activity of synthetic peptides against a panel of carbapenem-resistant enterococci (CRE) bacteria, including carbapenem-resistant *Klebsiella pneumoniae* (KpC+) and *Escherichia coli* (KpC+). Activity modulation depended on the type of ATCUN variant utilized. Membrane permeability assays revealed that the in silico selected lead template, CM15, and its ATCUN analogs increased bacterial cell death. Mass spectrometry, circular dichroism, and molecular dynamics simulations indicated that coordinating ATCUN derivatives with Cu(II) ions did not increase the helical tendencies of the AMPs. CM15 ATCUN variants, when combined with Meropenem, streptomycin, or chloramphenicol, showed synergistic effects against *E. coli* (KpC+ 1812446) biofilms. Motif addition also reduced the hemolytic activity of the wild-type AMP and improved the survival rate of mice in a systemic infection model. The dependence of these bioactivities on the particular amino acids of the ATCUN motif highlights the possible use of size, charge, and hydrophobicity to fine-tune AMP biological function. Our data indicate that incorporating metal-binding motifs into peptide sequences leads to synthetic variants with modified biological properties. These principles may be applied to augment the activities of other peptide sequences.
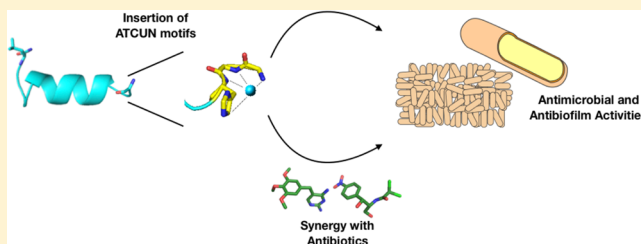
The emergence of resistant bacteria coupled to the decline in antibiotic innovation has led to an urgent need for alternatives to standard-of-care antibiotics, which often have limited efficacy.[1,2] Chronic and recalcitrant infections are on the rise, such as those caused by highly resistant Gram-negative bacteria. Carbapenem-resistant Enterobacteriaceae (CRE), in particular, may also be resistant to acylureidopenicillins, third generation cephalosporins, and fluoroquinolones.[3] In addition

to various antibiotic resistance mechanisms, CRE also deploy virulence factors, including the formation of biofilms.[4] Naturally occurring,[5,6] rationally designed,[7−9] or computationally generated[10−13] AMPs represent promising candidates to stand

against multiresistant bacteria because of their diversity, multifunctionality, and varied mechanisms of action.[14]

Bacterial biofilms[15] are responsible for ~80% of all nosocomial infections.[16] The communities of pathogenic microbes that form biofilms are associated with indwelling medical devices, including catheters, stents, and prosthetic implants. Bacterial biofilms represent a physiologically distinct growth state, with hundreds of genes changing expression compared to the expression profiles observed in bacteria grown under planktonic conditions.[17] These multicellular structures constitute an extracellular matrix, composed by extracellular polymeric substances (EPS) that protect the bacteria within the biofilm from exogenous agents such as antibiotics and constituents of the host immune system. EPS, a heterogeneous combination of polysaccharides, extracellular DNA (eDNA), proteins, and lipids held together by adhesins, forms an intricate network that immobilizes the microbes to the surface they colonize. Bacteria growing in biofilms cause chronic infections, which are extremely refractory toward even high concentrations of last resort antibiotics. These infections are, therefore, associated with high treatment costs and with high mortality and morbidity.[18] Unfortunately, no antibiofilm drug has yet been approved for clinical use[17,19−21] despite the importance of such drugs in combating biofilm-associated infections.

Several peptides have been described to date that are effective against biofilms[18,22−24] and that can synergize with conventional antibiotics and antifungal agents against drug-resistant organisms such as the ESKAPE pathogens (**E**nterococcus faecium, **S**taphylococcus aureus, **K**lebsiella pneumoniae, **A**cinetobacter baumannii, **P**seudomonas aeruginosa, and **E**nterobacter species).[22−26] Yet, although these peptides have shown great promise as novel antibiofilm agents for treating recalcitrant infections, significant hurdles have delayed their translation to the clinic.

Gram-negative bacteria such as CRE are among the most common pathogens found in biofilm-related infections. In fact, it is well established that carbapenamase-producing K. pneumoniae (KpC-Kpn) and multidrug resistant isolates of bacteria carrying the β-lactamase encoding genes bla_{PER-1} and bla_{VIM-2} have a high propensity to form robust biofilms on medical implants, such as urinary catheters.[27,28] Furthermore, Gram-negative bacteria may escape killing by AMPs because of the presence of lipopolysaccharide (LPS), which acts as a permeability barrier around the cell. Therefore, efforts are being made to design synthetic peptides that have enhanced activity against these hardy, highly resistant organisms.

Previous studies have described the concept of using catalytic metallodrugs as an effective strategy for improving the bioactivity of AMPs.[23,29−31] The amino terminal Cu(II) and Ni(II) (ATCUN) binding motif chelates metal ions and elicits the release of reactive oxygen species (ROS). The insertion of this motif at the N-terminal extremity of AMPs creates a molecular scaffold that catalyzes the formation of ROS, which is absent in the original AMP.[32−37] The presence of the ATCUN motif in many naturally occurring AMPs isolated from all branches of the phylogenetic tree indicates their potential as templates for the design of antimicrobial compounds (http://angeles-boza.chemistry.uconn.edu/atcun-amps/). The ATCUN motif shows high affinity for Cu(II) or Ni(II) ions (log $K \sim$ 14−15), forming with these ions a stable square pyramidal coordination complex.[38] The high affinity of ATCUN motifs for labile copper ions in bacteria that flow in response to environmental stimuli[39] suggests that the insertion of these

motifs within an AMP or an antibiofilm peptide (ABP) sequence would be likely to generate ROS in the surroundings of the bacterial cell membranes. The ATCUN-Cu(II) complex, in the presence of hydrogen peroxide and ascorbic acid, generates ROS via a Fenton-like reaction as the bound copper cycles between its +2 and +3 oxidation states.[40] This leads to ROS buildup at a high turnover rate, which can irreversibly degrade therapeutic targets such as nucleic acids and proteins, even when the AMP is present at subtherapeutic doses.[32,41] The ATCUN-AMP molecular scaffold has been exploited to degrade extracellular DNA (eDNA), one of the main components of EPS.[42] Degrading eDNA, which is considered a major target of antibiofilm agents,[43] may cause biofilms to disintegrate. We hypothesized that incorporating the ATCUN motif into AMPs would improve their direct killing of bacteria and also facilitate the ability of conventional antibiotics to disperse pre-existing biofilms and prevent new biofilm formation (Figure 1).
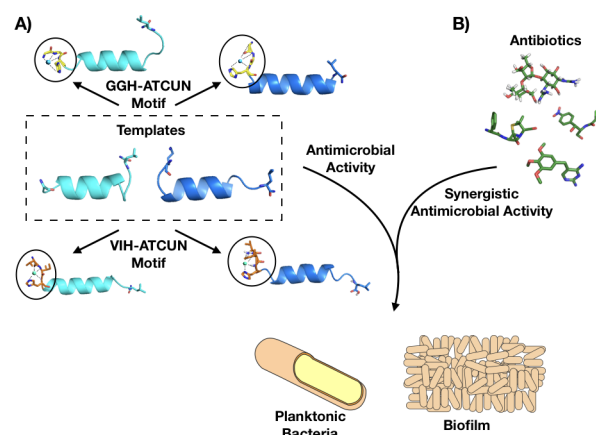

Figure 1. Design of ATCUN variants of the in silico selected templates. (A) ATCUN variants tested against planktonic bacteria in growth inhibition assays and against bacterial biofilms. (B) The synergistic effect of the lead ATCUN peptides with antibiotics against resistant bacteria.

■ METHODOLOGY

In Silico Selection of AMPs. We selected 14 cationic α-helical AMPs 10−20 amino acid residues long (Table S1) with broad-spectrum antimicrobial activity by using the search tool from the Antimicrobial Peptide Database (APD) (http://aps.unmc.edu/AP/database/antiA.php). We then modified the N-terminus of each AMP by adding the tripeptide motifs Gly-Gly-His (GGH) or Val-Ile-His (VIH), also known as the ATCUN motifs. A total of 28 ATCUN variants were generated and subjected to a three-step selection process. Each variant was submitted to two prediction modules, available at http://www.camp.bicnirrh.res.in/predict/[44] and http://www.biomedicine.org.ge/dbaasp/ (DBAASP - Database of Antimicrobial Activity and Structure of Peptides), to determine whether antimicrobial activity would be retained (Table S2). Peptides were ranked based on their likelihood of having antimicrobial activity, determined by whether the predictor returned a probability of <0.9 or "non-AMP" (Table S3).[45] Moreover, we initially built molecular models of ATCUN variants on the I-TASSER server[46] using a hierarchical approach for peptide structure prediction (as we could not determine reliable template structures for the comparative modeling of all peptides) to identify peptides whose α-helical structures were likely to be

disrupted upon the insertion of an ATCUN motif (Figure S1). Those not having this structure were ranked last (Table S3). Finally, the HeliQuest server (http://heliquest.ipmc.cnrs.fr/)[47] was used to generate helical wheel projections for all variants to study the properties of the hydrophobic and hydrophilic portions such as the polar angle and mean hydrophobic moment (Figure 2 and Figure S1).[48] We then ranked the 14 wild-type
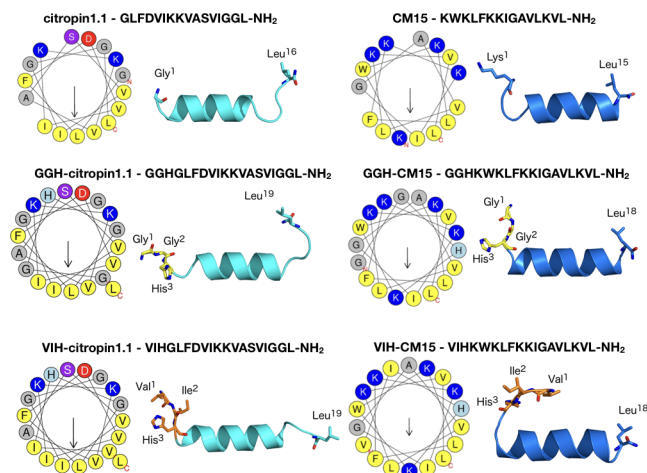

Figure 2. Helical wheel projections of the wild-type peptides and ATCUN variants. The vector of hydrophobic moment is shown as an arrow from the center of the helical wheel, while the estimated value of the resultant hydrophobic moment vector is proportional to the length of the arrow. Positively charged residues are indicated in blue; hydrophobic residues are indicated in yellow; residues with hydrophobicity close to zero are indicated in gray; and negatively charged residues are indicated in red. Three-dimensional theoretical structures for citropin1.1, GGH-citropin1.1, VIH-citropin1.1, GGH-CM15, and VIH-CM15 are obtained by comparative modeling. The NMR structure of CM15 in solution (PDB code: 2jmy) is also shown. Yellow sticks highlight the GGH-region, and orange sticks highlight the VIH-region.

AMPs as high or low based on the three-dimensional models generated by comparative modeling (SI Tables S2−S4). Each criterion was equally weighted. Based on the results, we selected CM15 and citropin1.1 from the top-ranked variants for chemical synthesis. Peptides were acquired from Biopolymers (MIT).

Mass Spectrometry (MS) Analysis of Copper Coordination. To determine whether the ATCUN variants of CM15 coordinated to Cu(II), peptides were analyzed using electrospray ionization mass spectrometry on a 4,000 Q-Trap mass spectrometer at room temperature and with a cone voltage of 5 kV. The peptides were diluted in a solution containing 47.5% H_2O, 47.5% acetonitrile, 5% dimethyl sulfoxide, and 0.1% formic acid to a concentration of 50 μM. The Cu(II)-containing samples were incubated with 0.9X $CuCl_2$ for 30 min prior to injection in the mass spectrometer.

In Vitro Growth Inhibition Assays. The following strains were used in antimicrobial susceptibility assays: *E. coli* (ATCC 25922), *E. coli* MG1655, *E. coli* (KpC+ 1812446), *E. coli* (KpC+ 2101123), *K. pneumoniae* (KpC+ 1825971), *K. pneumoniae* (ATCC 13883), *P. aeruginosa* (ATCC 27853), methicillin-resistant *S. aureus* (3730529), and *S. aureus* (ATCC 25923). Antimicrobial susceptibility tests were performed using the broth microdilution method.[38] Minimum inhibitory concentration (MIC) was defined as the minimal 100% inhibitory concentration of peptide after 12 h of incubation at 37 °C. To

study the influence of Cu(II) ions on the antimicrobial activity of ATCUN peptides, Mueller Hinton (MH) broth was supplemented with $CuSO_4 \cdot 5H_2O$ to achieve a final concentration of 0.25 mM of Cu(II) ions, below the toxic level (7.8 mM) against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) determined in our laboratory. As controls, the cell-impermeable Cu(II) chelator triethylenetetramine (TETA) and the cell-permeable Cu(II) chelator tetrathiolmolybdate (TTM) were used to determine the importance of Cu(II) in antimicrobial activity. The chelators at a concentration of 200 μM were incubated with *E. coli* MG1655 for 10 min prior to exposure to the peptide solutions at room temperature. All bacterial cell cultures used for these experiments were in exponential growth phase.

Cell Permeability Assays. Confocal fluorescence microscopy was used to observe the permeabilizing effect of CM15 and its ATCUN-derivatives on *E. coli* membranes. Briefly, *E. coli* MG1655 was treated with the peptides at their MICs for 1 h at 37 °C in a shaking incubator. The cell membranes were stained for 30 min with FM4-64 and SYTOX green, a cell-impermeable dye. The bacteria were then spotted onto agarose pads on glass microscope slides and covered with a glass coverslip. Cells were imaged using a Nikon A1R spectral confocal microscope with a 60× oil immersion lens. Images were analyzed using ImageJ 1.8.0.

Inhibition of Bacterial Growth and Ability To Form Biofilm Determined by Crystal Violet Quantification Assay. Inhibition of biofilm formation by the peptides was assessed against susceptible and carbapenem-resistant *E. coli* and *K. pneumoniae* in BM2 minimal medium [62 mM potassium phosphate buffer, pH 7.0, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 mM $FeSO_4$, 0.5% glucose] using methods described by Wiegand et al.[49] Planktonic cell growth was determined by measuring absorbance at 600 nm at the end of the incubation period in the presence of peptide that was added at the beginning of the experiment, and biofilm formation was assessed using the crystal violet (CV) assay for biofilm biomass quantification. CV binds to negatively charged bacteria and to polysaccharides of the EPS. The amount of CV adsorbed is directly proportional to the biofilm biomass.[50] Biofilms were stained with 0.1% CV solution (100 μL per well) for 20 min at room temperature. The plates were washed three times by flooding with double distilled water and thoroughly dried by tapping onto paper towels several times followed by air drying. The bound CV was solubilized with 95% ethanol (110 μL per well) for 10 min, and the absorbance of extracted CV was measured at 595 nm.[51]

Checkerboard Assay To Evaluate Synergistic Interactions between Peptides and Antibiotics. The synergistic effects between CM15 or its ATCUN variants and five antibiotics were assessed by checkerboard titration in a 96-well microplate. The antibiotics ampicillin, trimethoprim, Meropenem, streptomycin, and chloramphenicol were used in this study because they act by different mechanisms of action. The synergistic interactions in the growth inhibition assays were expressed as the fractional inhibitory concentration index (FICI)

$$\text{FICI} = \left(\frac{[A]}{\text{MIC}_A}\right) + \left(\frac{[B]}{\text{MIC}_B}\right)$$

where MIC_A and MIC_B are the MIC values of antibiotic (A) and peptide (B) solely, and [A] and [B] are the MIC values of A and B in combination. The FICI values were interpreted as follows:

FICI ≤ 0.5 was considered a synergistic effect, 0.5 < FICI ≤ 1 was considered an additive effect, and 1 < FICI ≤ 2 was considered an indifferent effect. The same method was used to calculate the synergistic effect in the antibiofilm assays but using the minimal biofilm inhibition concentration (MBIC), which was the minimum concentration of antimicrobial agent that prevents biofilm formation ($MBIC_{100}$ for 100% inhibition). All FICI values were determined by the lowest $MBIC_{100}$ value (e.g., for >32 μM, 64 μM was used).

Structure Analysis. Circular dichroism (CD) spectra of the peptide solutions at 50 μM were obtained in the presence and absence of Cu(II) ion, on a Jasco J-710 spectropolarimeter in the far UV region with a quartz cuvette with a path length of 1 mm at room temperature, over five accumulations and a scan rate of 10 nm/min. The experiments were performed in a 50:50 mixture of nanopure water and 2,2,2-trifluoroethanol (TFE), a well-known helical inductor.[52] Molar ellipticity and α-helical content were calculated via established methods.[53]

Comparative Modeling and Molecular Dynamics (MD) of Lead AMPs. Prior molecular dynamics (MD) simulations in TFE for the three-dimensional theoretical models for lead peptides, CM15 variants (GGH and VIH) and citropin1.1 (parental; GGH and VIH), were built using Modeler v. 9.17[54] based on the solution NMR structure of CM15 in DPC micelles (PDB code: 2jmy),[55] as well as on the solution NMR structure of Aurein 1.2 (PDB code: 1vm5 − template identified by BLASTp analysis), respectively. CM15 (parental) was not modeled as its atomic coordinates are available in the Protein Data Bank (PDB code: 2jmy); these known coordinates were used for further in silico analysis. A total of 100 structures were generated, and the lowest free-energy models for all the peptides were selected for validation procedures, including PRO-CHECK[56] for stereochemistry evaluation and ProSA-web[57] for fold quality check. Structure visualization was done in PyMOL (http://www.pymol.org). MD simulations for the validated models and CM15 (PDB code: 2jmy) were conducted in 50% TFE/water (v/v - molar ratio 1:4), according to Cardoso et al.,[58] using the single point charge (SPC) water model. All simulations were carried out in cubic boxes using the GROMOS96 43A1 force field and the computational package GROMACS v.5.0.4.[59] Chloride ions (Cl^-) and sodium ions (Na^+) were used to neutralize the charges of the systems. Geometry of water and molecules was constrained using the SETTLE algorithm.[60] The LINCS algorithm was used to link all the atom bond lengths. Particle Mesh Ewald (PME) was used for electrostatic corrections (radius cutoff of 1.4 nm). The same radius cutoff was also used for van der Waals interactions. The list of neighbors of each atom was updated every 5,000 simulation steps of 2 fs each. The steepest descent algorithm (50,000 steps) was applied for energy minimization. After that, the systems underwent a normalization of temperature and pressure to 300 K and 1 bar, using the velocity rescaling thermostat (NVT) and the Parrinello−Rahman barostat (NPT), respectively, for 100 ps. The systems with minimized energy and balanced temperature and pressure were submitted to MD simulation for 100 ns. The root-mean-square deviation (RMSD) and root-mean-square fluctuation were analyzed for each simulation. Three independent simulations were performed for each system.

Hemolytic Assay. Hemolytic assays were conducted to assess the suitability of peptides for in vivo trials and selectivity toward bacterial membranes. Hemolysis was measured for red blood cells (RBCs) from mice (approved by the Ethics Committee of Universidade Católica Dom Bosco number 019/2016). Fresh blood was collected into EDTA-coated vacutainers and washed three times with sterile PBS. A small aliquot of washed cells was resuspended in fresh PBS to make a 0.8% (v/v) solution of RBCs. Then a 75 μL aliquot of RBCs was mixed with a 75 mL aliquot of a 2-fold serial dilution series of the peptides, and the mixture was incubated at 37 °C for 1 h in polypropylene PCR tubes. Triton X-100 and PBS were used as positive and negative controls, respectively. After incubation, the tubes were spun down at 4,400 rpm at 4 °C for 10 min, and 100 mL of the supernatant was transferred into a clear 96-well plate. The absorbance at 414 nm was measured and normalized against the absorbance of the positive and negative controls. Data were obtained from four independent trials and presented as the mean ± standard deviation.

In Vivo Assays. Male Balb/C (18−20 g) mice from the Biotério Central do Campus da USP in Ribeirão Preto, São Paulo, were kept in groups of 5 per cage at 22 °C with normal cycles of light and free access to food and water. The care and use of the animals were approved by the Ethics Committee of Universidade Católica Dom Bosco number 019/2016. We assessed the ability of the peptides to protect the mice from lethal systemic infections induced with *E. coli* KpC+ 1812446. The mice (n = 40) were randomly placed into eight groups of five mice each, and each mouse was injected intraperitoneally with 200 μL of saline solution containing 2×10^7 CFU of *E. coli* KpC+ 1812446. Intraperitoneal treatment with 5 or 10 mg·kg^{-1} of each peptide was initiated after 1 h of infection and repeated at 24 h intervals for 7 days.[61] Gentamicin at 10 mg·kg^{-1} of body weight and normal saline were used as positive and negative controls, respectively.

Statistical Analysis. The GraphPad Prism v6.0 software (Software GraphPad, USA) was used for the Mann−Whitney tests and to determine the significance of the mortality rate among the experimental groups, using the Kaplan−Meier test as the statistical parameter. Data were expressed as mean ± SD of all sample groups.

■ RESULTS

In Silico Selection of CM15 and Citropin1.1. As initial selection criteria, the length, cationicity, helicity, and spectrum of activity were used to narrow down a list of potential AMPs. We then selected CM15 and citropin1.1, two known pore-forming AMPs,[62,63] for further studies based on 1) prediction algorithms considering the effect of the insertion of ATCUN motifs on the helical tendency, 2) analyses of the helical wheel projections for ATCUN-AMP variants, and 3) molecular modeling of the three-dimensional structures (Figures 2, S2, and S3; Tables S1−S4).

CM15 and citropin1.1 were modified by using two well-known ROS-generating ATCUN motifs: Gly-Gly-His (GGH) and Val-Ile-His (VIH). These motifs, which had been reported by Libardo et al.,[64] do not occur naturally but were rationally designed according to the general NH_2-XXH ATCUN motif sequence, where X is any canonical amino acid except proline, and H is histidine, which must always be in the third position of the N-terminal extremity. ROS generation confers cytotoxic activity to ATCUN motifs in the presence of Cu(II) ions via Fenton-like reactions.[65] CM15, citropin1.1, and their ATCUN variants were synthesized by solid-phase peptide synthesis and a fluorenylmethyloxycarbonyl strategy by AminoTech (São Paulo, Brazil). Model molecules and the motifs GGH and VIH were used as controls.

Antimicrobial Activity of Templates and ATCUN-AMPs. We performed antimicrobial susceptibility assays against Gram-negative and Gram-positive bacteria susceptible and resistant to carbapenem, as previously described.[66] Table 1

Table 1. Minimum Inhibitory Concentration (MIC) of CM15, Cit1.1, and Their ATCUN Variants against Pathogenic Bacteria in the Absence and Presence of Cu(II) Ions

bacteria	MIC (μM)[a]					
	CM15	GGH-CM15	VIH-CM15	Cit1.1	GGH-Cit1.1	VIH-Cit1.1
E. coli (ATCC 25922)	2	2	1	10	34	>33
E. coli (KpC+ 1812446)	2	2	4	10	34	33
E. coli (KpC+ 2101123)	4	2	2	5	9	16
K. pneumoniae (KpC+ 1825971)	16	2	1	20	>34	>33
K. pneumoniae (ATCC 13883)	2	1	1	20	34	>33
P. aeruginosa (ATCC 27853)	2	1	4	>40	>34	>33
MRSA (3730529)	4	2	2	10	17	16
S. aureus (ATCC 25923)	2	1	1	3	9	4
MIC (μM) in Cu(II) supplemented medium (0.25 μM)						
E. coli (ATCC 25922)	2	2	1	10	34	>33
E. coli (KpC+ 2101123)	4	2	2	5	9	16
K. pneumoniae (KpC+ 1825971)	16	2	1	20	>34	>33
S. aureus (ATCC 25923)	2	1	1	3	9	4

[a]MIC is defined as the lowest concentration required to inhibit visible growth of bacteria, confirmed by optical density at 600 nm (OD_{600}). Data correspond to the mean of three independent experiments.

summarizes the activity of peptides CM15 and citropin1.1 with and without the incorporation of the ATCUN motif and with and without exposure to Cu(II) ions. These data indicate that, for some bacterial strains, the antimicrobial activity of CM15 was modulated by incorporation of the ATCUN motif: the potency of the GGH and VIH variants of CM15 against carbapenem-resistant *K. pneumoniae* (KpC+ 1825971), for example, was 4-fold and 8-fold higher, respectively, than the potency of the original peptide. The antibacterial activity of CM15 against the carbapenem-resistant strains *E. coli* (KpC + 1812446), *E. coli* (KpC + 2101123), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC27853) was marginally improved by the presence of the ATCUN motif (Tables S5 and S6). This small improvement is nevertheless significant considering that pulmonary infections caused by carbapenem-resistant *K. pneumoniae* (KpC+ 1825971) have a mortality rate on the order of 40%.[67] However, ATCUN modification lowered the antimicrobial activity of citropin1.1, resulting inasmuch as a 3-fold decrease in antimicrobial activity against the Gram-negative and Gram-positive bacteria tested (Table 1). As ATCUN motifs require Cu(II) ions for their catalytic activity, we supplemented the growth medium with 0.25 μM Cu(II) solution. Antimicrobial activities obtained after the addition of Cu(II) ions were not changed when compared to those assayed in MH broth only (Table 1). The lack of enhanced activity upon the addition of

Cu(II) underscores the ability of ATCUN-AMPs to scavenge labile Cu(II) ions from the media or the bacteria themselves.[64]

ATCUN-CM15 Peptides Enhance Their Antimicrobial Activity in the Presence of Copper Ions. MIC values were also obtained in the presence of Cu(II) chelators to determine the importance of the concentrations of trace Cu(II) ions found in the growth medium used to evaluate the antimicrobial activity of the peptide CM15 and its ATCUN variants. Table 2 shows

Table 2. MICs of CM15 and Analogs against *E. coli* MG1655 in the Presence and Absence of Copper Ion Chelators TTM and TETA

peptide	no chelator (μM)	TTM (μM)	TETA (μM)
CM15	1	32	1
GGH-CM15	1	32	2
VIH-CM15	0.5	32	1

that the activity of the ATCUN peptides decreased 2-fold in the presence of the cell-impermeable Cu(II) chelator triethylene-tetramine (TETA), indicating that GGH-CM15 and VIH-CM15 require Cu(II) for their observed activity; whereas the activity of the wild-type peptide, which remained unchanged, is independent of the presence of Cu(II) ions. In contrast, addition of the cell-permeable Cu(II) chelator tetrathiolmolybdate (TTM) led to smaller MIC values for all three peptides, likely the result of ionic interactions between the negatively charged chelator and the cationic peptides, decreasing the overall availability of the peptides. However, the GGH-CM15 and VIH-CM15 variants were more strongly affected than CM15, showing that the competition for Cu(II) ion chelation by TTM influences the antimicrobial activity of the ATCUN motif-containing peptides.

Furthermore, mass and CD spectroscopy data (Figure S4A–C) coupled to MD simulations (Figures S2 and S3) confirmed that the addition of the ATCUN motifs to the original peptide sequence as well as the formation of the Cu(II) ion-ATCUN motifs complex did not interfere with the helical content of the AMPs compared to the template peptide CM15. Changes in the helical content were calculated using CD spectrometry assays, which revealed small variations on helical tendency compared to the wild-type peptide CM15 (>5%).

CM15 and Its Derivatives Permeabilize Bacterial Membranes. In order to determine whether the addition of the ATCUN motif results in any major changes to the activity of CM15, we used confocal fluorescence microscopy to observe possible morphological damage in *E. coli* MG1655 treated with the peptides. The cells were first treated for 1 h with one of the three peptides (CM15 and the GGH-CM15 and VIH-CM15 variants), followed by staining with FM4-64 to label the bacterial membranes and SYTOX Green, a cell-impermeable dye. Figure 3A shows confocal microscopy images of bacteria treated with CM15, GGH-CM15, and VIH-CM15. Phenotypic analysis of all of the samples presented damaged membranes, as indicated by the presence of SYTOX green staining, whereas the untreated control exhibited intact cell membranes. Moreover, average SYTOX Green intensity was measured (Figure 3B) to determine the relative extent of membrane permeation. Both wild-type CM15 and GGH-CM15 showed similar levels of intracellular SYTOX Green, whereas VIH-CM15 showed lower levels of intracellular SYTOX Green. These data indicate that all peptides damaged the integrity of bacterial membranes, although CM15
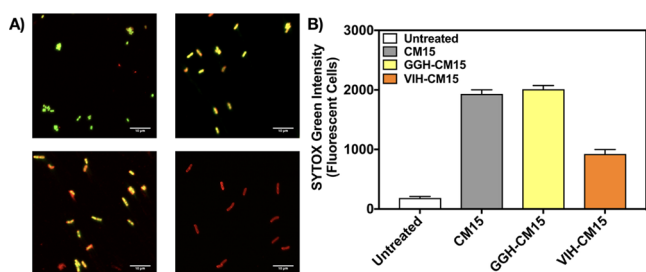
Figure 3. (A) *E. coli* treated with CM15 (upper left quadrant), GGH-CM15 (upper right quadrant), or VIH-CM15 (lower left quadrant), and untreated (lower right quadrant). (B) The average intensity of SYTOX Green in *E. coli* cells treated with CM15, GGH-CM15, and VIH-CM15. Error bars represent standard deviation.

and GGH-CM15 caused more membrane damage than VIH-CM15.

ATCUN Motif Enhances Synergy between CM15 and Antibiotics against Biofilms. A sought-after feature of AMPs and ABPs is their potential ability to enhance the antimicrobial and antibiofilm activity of conventional antibiotics, which for some peptides has been demonstrated previously. AMPs might enhance the sensitivity of resistant bacteria to the conventional antibiotic or decrease cross-resistance to both AMPs and antibiotics.[68] We tested whether the peptides CM15 and its GGH- or VIH-variants changed the activity of several antibiotics: Meropenem, ampicillin, trimethropim, streptomycin, and chloramphenicol. Results of the checkerboard assays for the inhibition of biofilm formation and the growth of planktonic cells for each combination are presented in Table 3. Results for each peptide and antibiotic combination are provided in Tables 3, Tables S7, and S8

Our data indicate that the ATCUN motifs had influence on the antibiofilm activity of antibiotics used in the checkerboard assays in a manner highly dependent on the type of ATCUN motif (GGH or VIH), the growth phase of the bacteria (whether in biofilm or planktonic phase), and the mechanism of action of the antibiotic. Biofilm formation by carbapenem-resistant *E. coli* (KpC+ 1812446) was completely prevented with a combination of Meropenem and either the parental peptide CM15, GGH-CM15, or VIH-CM15 (Table S7). The same combinations, however, were ineffective at inhibiting carbapenem-resistant *E. coli* (KpC+ 1812446) planktonic cells (Table S8). Complete inhibition of the proliferation of carbapenem-resistant *E. coli* (KpC+ 1812446) in the planktonic phase was observed only when the peptides were combined with chloramphenicol or

streptomycin (Table 3). This variance in effect among several conventional antibiotics in combination with the same peptide highlights possible differences in the mechanisms that inhibit biofilm formation or prevent the growth of planktonic cells. There may also be differences in defense mechanisms deployed by *E. coli* (KpC+ 1812446). Planktonic cell growth was inhibited by streptomycin only in combination with VIH-CM15, clearly pointing to the importance of the ATCUN motif to the outcome of combinations. The synergies with FICI of 0.33, 0.27, and 0.185, for the combination of Meropenem with CM15, GGH-CM15, and VIH-CM15, respectively (Table 3), translate into a 32-fold (Meropenem and GGH-CM15) and 16-fold (Meropenem and VIH-CM15) decrease in the concentration of Meropenem required to completely inhibit *E. coli* (KpC+ 1812446) biofilm formation, compared to a 4-fold reduction for meropenen and CM15 wild-type (Table 3). The drastic reduction in MICs and MBICs has far reaching implications for the treatment of infections caused by carbapenem-resistant *E. coli* (KpC+ 1812446) and is clearly dependent on the type of ATCUN motif conjugated to CM15 (Tables S5 and S6). It is also interesting to note that, although some combinations were not synergistic, the presence of the ATCUN motifs nevertheless enhanced the activity of the antibiotic, resulting in a marginal reduction in the concentration required to prevent biofilm formation. This was the case when CM15 or its variants were combined with streptomycin or trimethoprim, which reduced the concentration of antibiotic needed to inhibit biofilm formation by 4-fold (FICI > 0.5) (Table 3). Similarly, the combination of trimethoprim with CM15, GGH-CM15, or VIH-CM15 inhibited the growth of planktonic cells by 8-fold, 4-fold, and 2-fold, respectively, compared to the activity of the antibiotic alone (Table 3). The wild-type CM15 and VIH motif appear to enhance antibiotic action most in the biofilm phase, whereas the influence of the GGH motif in antibiotic synergy is more pronounced in the planktonic phase (Table 3).

Hemolytic Activity of CM15 and Citropin1.1 Is Lowered by ATCUN Modification. The hemolytic activity of the ATCUN variants was reduced as compared with that of the parent peptides at the MIC value for *E. coli* KpC+ (Table 4). This reduced hemolysis suggests that AMPs that have incorporated an ATCUN motif may be less harmful to mammalian cells than AMPs lacking the motif.

The VIH-ATCUN Motif Incorporated into the CM15 Sequence Led to Higher Survival in a Mouse Infection Model. We assessed the ability of the peptides to protect mice in a systemic *E. coli* KpC+ 1812446 infection model. Survival at 24 h intervals for 1 week is presented in Figure 4A−C. A single dose

Table 3. Synergistic Effect of CM-15 and Its Variants with Meropenem, Ampicillin, Trimethoprim, Chloramphenicol, and Streptomycin on the Inhibition of Planktonic and Biofilm Formation by *E. coli* (KpC+ 1812446)[a]

peptide	FICI					antibiotic MIC decrease (fold)				
	MER	AmP	TRI	CHL	STR	MER	AmP	TRI	CHL	STR
Planktonic										
CM15	1.4	1.8	0.525	0.8	0.69	-	-	8	**495**	4
GGH-CM15	0.52	1.52	0.5	**0.5**	1.5	4	-	4	132	-
VIH-CM15	1.0	2.0	1.0	1.0	1.29	2	-	2	495	2
Biofilm										
CM15	**0.33**	1.2	2.0	2.0	0.5	4	-	-	-	4
GGH-CM15	**0.27**	2.0	1.5	2.0	0.78	32	-	2	-	4
VIH-CM15	**0.185**	1.5	1.0	1.38	1.0	16	-	2	-	2

[a]Boldface fractional inhibitory concentration indices (FICIs) indicate synergy. Hyphen (-) indicates no change in antibiotic concentration.

Table 4. Hemolytic Activity of the Lead Peptides on Mouse Red Blood Cells*[a]*

peptide	hemolysis (%)
CM15	40 ± 5
GGH-CM15	29 ± 2
VIH-CM15	29 ± 3
citropin1.1	16.5 ± 3
GGH-citropin1.1	0.2 ± 0.05
VIH-citropin1.1	0.9 ± 0.1

*[a]*The highest concentration (μM) used was based on the MIC of peptides against *E. coli* KpC+ 1812446. The values are represented as mean ± standard deviation of three independent experiments.
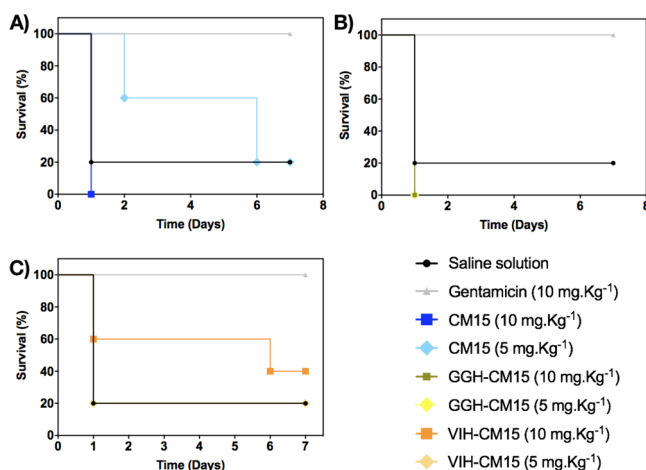

Figure 4. Protective activity of CM15, GGH CM15, and VIH CM15 in animal models of systemic infection. Each mouse ($n = 5$) received intraperitoneally 2×10^7 CFU *E. coli* (KpC+ 1812446) in a total volume of 200 μL. After 1 h, treatments were started with the peptides (A) CM15 (5 and 10 mg·kg^{-1}), (B) GGH-CM15 (5 and 10 mg·kg^{-1}), (C) VIH CM15 (5 and 10 mg·kg^1), and gentamicin (10 mg·kg^{-1}; positive control) or sterile physiological solution (negative control). The treatment was performed daily, and the survival curve was evaluated for 7 days.

of CM15 (5 mg·kg^{-1}) prolonged mouse survival 2-fold, compared to the untreated control after 7 days; however, doses of 10 mg·kg^{-1} led to mouse deaths after 2 days of treatment. The literature is not consistent regarding CM15 toxicity; hemolytic activity has been described as negligible,[69] but a recent study reported high toxicity.[70] Our results showed high in vivo toxicity of CM15. The insertion of the GGH motif did not decrease the toxicity of CM15 at 5 and 10 mg·kg^{-1} (all the mice died on the first day). The insertion of the VIH motif in the sequence decreased the toxicity of CM15. Remarkably, VIH-CM15 at 10 mg·kg^{-1} showed similar levels of protection from *E. coli* KpC+ 1812446 infection as CM15 at 5 mg·kg^{-1}, protecting 60% of the mice after the first day and 40% at the sixth and seventh days.

■ DISCUSSION

Novel strategies are needed to combat antibiotic-resistant infections. Here, we designed dual-acting catalytic metallodrugs that exploit the bioinorganic chemistry of the ATCUN motif to directly kill bacteria and modulate biofilm formation by CRE. This motif displays nanomolar to picomolar affinities toward copper and nickel ions, while the coordination with other metals is at least 3 orders of magnitude lower.[71] Thus, copper and nickel

are excellent candidates for designing peptides that bind specifically to them. The composition and geometrical disposition of these motifs present much higher affinity toward copper and nickel ions when these metal ions are in their second oxidation state as expected for their position within the Irving-Williams series. Here, we particularly described the complexation of ATCUN motifs with copper, as this metal is mobilized in response to bacterial infections. Importantly, copper concentrations have been shown to increase in urine during urinary tract infections,[72] as well as serum and liver.[73] In addition, copper levels are also elevated within macrophages where concentrations above 500 μM have been detected.[74]

The concept of catalytic metallodrugs, although in its nascent stages, has been applied experimentally to enhance the potency of AMPs across the spectrum of molecular mechanisms of antibacterial activity. Unlike conventional AMPs, the ATCUN motif-containing AMPs generate cytotoxic chelates in the presence of Cu(II) ions,[75] thereby disrupting bacterial cell membranes, interfering with DNA,[41] and enhancing the activity of the AMPs.

Our data confirm earlier reports that the ATCUN motif can be used to improve the bioactivity of short cationic α-helical AMPs (e.g., CM15) against CRE pathogens, including *K. pneumoniae* (KpC+ 1825971), which is known to cause fatal lung infections in patients in intensive care units.[76] Interestingly, the increase in bioactivity is ATCUN motif dependent. We observed that the GGH and VIH motifs resulted in an 8-fold and 4-fold increase in the activity of CM15 against *K. pneumoniae* (KpC+ 1825971), respectively. This improvement suggests that the bioactivity of the ATCUN-AMP complexes can be fine-tuned by varying the polarity, hydrophobicity, or size of the amino acids that make up the motif and highlights the potential use of catalytic metallodrugs for treating multidrug-resistant bacteria. These results also suggest the value of analyzing a large number of strains, to determine whether the improvement in antibacterial activity extends over a large range of pathogens.

CD studies performed on CM15 and its ATCUN variants showed that the addition of the ATCUN motif to the wild-type peptide leads to a 4−12% increase in α-helicity in an ATCUN motif-dependent manner. These findings were also observed in our MD simulations, in which both variants showed preserved or slightly increased helical content after 100 ns of simulation. The higher helical content may have slightly increased the antimicrobial activity of the ATCUN peptides compared to that of the wild-type peptide CM15. When the peptides were studied in the presence of Cu(II), the α-helical content decreased slightly, showing that upon binding Cu(II) there is a slight deformation of the secondary structure of the peptide. These results indicate that the ATCUN motif confers greater activity to CM15 through a combination of an increase in the overall α-helicity and the generation of ROS as a result of the binding of Cu(II).[64] Electrospray Ionization-Mass Spectrometry (ESI-MS) data confirmed that the ATCUN-CM15 peptides bind to Cu(II) ions. CM15 without an ATCUN motif did not bind to Cu(II), whereas both GGH-CM15 and VIH-CM15 showed the presence of bound and unbound copper species, with each ATCUN peptide capable of binding to only one copper ion (Figure S4).

These results suggest that there is no need to incorporate Cu(II) metals within the ATCUN-AMP complex drugs since the motif is able to scavenge metal ions in the plasma or target organism. This should avoid many regulatory bottlenecks associated with the use of metals in drugs.[71]

ATCUN motifs are also able to affect the antibiofilm activity of conventional antibiotics, most of which cannot target biofilms even at concentrations many fold above their MICs.[25,77] In the present study, ATCUN variants of CM15 interacted with Meropenem, chloramphenicol, and streptomycin to counter biofilms (Tables S7 and S8). This result is particularly interesting considering that Meropenem (a carbapenem) is among the antibiotics of last resort whose usage is restricted to the treatment of highly recalcitrant infections. Therefore, resistance to this class of antibiotics by CRE is extremely worrisome. In these cases and especially when biofilm formation is implicated, higher concentrations of the antibiotic are administered but may still fail to clear the infection.[78] The role played by the ATCUN motif in determining the extent of synergy with Meropenem, for example, offers several options for bioinorganic chemists to fine-tune this modulation by slight alterations of the amino acids. This singular feature of "tunability" has not been possible with traditional carbon–carbon (C–C) chemistry.

A common drug resistance mechanism, especially in Gram-negative bacteria, is the modification of the outer membrane, which limits the influx or uptake of toxic compounds, thereby preventing drugs from reaching their intracellular target.[79] If CM15 damages the membrane, either through the creation of transient pores or through a carpet mechanism, it might facilitate Meropenem translocation, allowing the antibiotic access to the cell wall, where it inhibits cell wall biosynthesis. The oxidative power of the ATCUN-CM15 derivatives may increase the duration of the transient pores,[80] resulting in more efficient transport of Meropenem. Results such as these could be useful for the future development of combination therapies. In fact, many AMPs create transient pores in the bacterial membrane.[19] It would be of interest to determine whether the ATCUN motif enhances antibacterial activity by facilitating membrane damage by the peptide and how this mechanism could be tuned.

Although little is yet known about the ATCUN motif beyond its interaction with Cu(II) and Ni(II) ions and the requirement of this interaction for the observed antimicrobial activity, the results of this study point to a dual role (antimicrobial and antibiofilm) of ATCUN-AMPs in defense against invading pathogens. Additionally, our results demonstrate that these modified peptides also have synergistic effects with some antibiotics. The insertion of ATCUN motifs, therefore, offers a promising strategy for the generation of potent broad-spectrum AMPs.

CONCLUSIONS

We have demonstrated that conjugating ATCUN motifs to AMPs represents a simple strategy for modulating antibacterial and antibiofilm activities. Further studies should focus on the structure–function relationships that contribute to these activities. Two wild-type AMPs, CM15 and citropin1.1, were selected from an initial set of 14 AMPs using criteria based on prediction algorithms, molecular modeling, and helical wheel projections. The antimicrobial assays indicated that the antibacterial activity, especially against the lung pathogen *K. pneumoniae* (KpC+ 1825971), was influenced by the type of ATCUN motif. Results from confocal fluorescence microscopy experiments with SYTOX Green confirmed the influence of the ATCUN motif in membrane permeabilization. Checkerboard assays to determine synergistic interactions between AMPs and antibiotics indicated that CM15 or its ATCUN variants enhanced the activity of conventional antibiotics such as

Meropenem, chloramphenicol, and streptomycin against *E. coli* (KpC+ 1812446) biofilms. Considering the ubiquitous nature of ATCUN-conjugated AMPs across the phylogenetic tree and the ability of this motif to modulate several biological functions, we speculate that ATCUN motifs may play a role in natural host defense mechanisms, which can be exploited to develop antimicrobial agents. In conclusion, converting AMPs into catalytic metallodrugs represents a promising strategy to combat antibiotic resistance, and additional studies are warranted to fully elucidate the mechanisms of action and potential therapeutic applications of ATCUN-containing AMPs.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.9b00440.

> List of helical peptides; prediction algorithm output data; helical wheel projections; peptide ranking for motif insertion; theoretical models; molecular dynamics simulations; circular dichroism spectra; helical content; characterization; minimal inhibitory concentration; minimal biofilm inhibitory concentration; and fractional inhibitory concentration index (PDF)

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