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Copper-binding tripeptide motif increases potency of the antimicrobial peptide Anoplin via Reactive Oxygen Species generation

M. Daben J. Libardo, Sai Nagella, Andrea Lugo, Scott Pierce, Alfredo M. Angeles-Boza *

Department of Chemistry, University of Connecticut, Storrs, CT 06269, USA

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ABSTRACT

Antimicrobial peptides (AMPs) are broad spectrum antimicrobial agents that act through diverse mechanisms, this characteristic makes them suitable starting points for development of novel classes of antibiotics. We have previously reported the increase in activity of AMPs upon addition of the Amino Terminal Copper and Nickel (ATCUN) Binding Unit. Herein we synthesized the membrane active peptide, Anoplin and two ATCUN-Anoplin derivatives and show that the increase in activity is indeed due to the ROS formation by the Cu^{II}-ATCUN complex. We found that the ATCUN-Anoplin peptides were up to four times more potent compared to Anoplin alone against standard test bacteria. We studied membrane disruption, and cellular localization and found that addition of the ATCUN motif did not lead to a difference in these properties. When helical content was calculated, we observed that ATCUN-Anoplin had a lower helical composition. We found that ATCUN-Anoplin are able to oxidatively damage lipids in the bacterial membrane and that their activity trails the rate at which ROS is formed by the Cu^{II}-ATCUN complexes alone. This study shows that addition of a metal binding tripeptide motif is a simple strategy to increase potency of AMPs by conferring a secondary action.

1. Introduction

The rise of bacterial resistance against small-molecule antibiotics is a growing global health problem that has led to increased interest in the discovery of agents with a unique mode of action. Due to their broad-spectrum activity, Antimicrobial peptides (AMPs) have arisen as a paradigm for the design of novel antibiotics. AMPs are a component of the innate immune system of many organisms, from bacteria and fungi to vertebrates, invertebrates and plants [1–3]. These peptides are typically short and cationic, although many anionic AMPs have been identified [3]. AMPs have membrane solubilizing, cell penetrating and DNA/RNA binding abilities [4,5]. Numerous peptide-small molecule conjugates have been synthesized to improve the efficacy of AMPs. Conjugates relevant to this work include amine-based metal binding groups [6], porphyrins [7–9], and chromophores [10–12]. The latter two conjugate types are dual acting in that they generate Reactive Oxygen Species (ROS) in addition to their classical antibiotic action. The resulting ROS renders the bacteria more susceptible to the conjugates.

The naturally occurring Amino Terminal Copper and Nickel (ATCUN) Binding Motif with the consensus sequence H_2N-Xaa-Xaa-His has high affinity for Cu^{2+} and Ni^{2+} ions [13]. Decades of research have shown that the activity of the ATCUN motif is not limited to transport of metals, but it can also have other potential biological functions. For example, the human protamine P2 (HP2), an important protein involved in the production and maturation of sperm, contains the ATCUN sequence Arg-Thr-His at its N-terminus [14]. HP2 has been suggested to play a major role in sperm DNA damage due to its ability to affect proper DNA assembly and generate ROS upon metal binding. Model peptides have shown that the damage caused by HP2 is linked to the presence of the ATCUN sequence [14]. In addition, numerous studies have shown that the Cu^{II}-ATCUN complex has nuclease and protease activity owing to the fact that it can form Reactive Oxygen Species [15–19].

Naturally occurring peptides with antimicrobial activity containing an ATCUN motif have also been reported, although whether the motif is biologically relevant or not is yet to be determined. The amino acid sequence of the human salivary peptide histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY) and other histatins contain an ATCUN motif. Notably, the antifungal activity of histatin 5 has been related to the ROS generating activity of the Asp-Ser-His motif [20–24]. Similarly, Myxinidin (GIHDILKYGKPS), an AMP derived from the epidermal mucus of hagfish, contains the ATCUN

* Corresponding author.
E-mail address: alfredo.angeles-boza@uconn.edu (A.M. Angeles-Boza).

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sequence Gly-Ile-His [25]. Interestingly, when the histidine residue is replaced with a lysine, the peptide becomes less active against *Escherichia coli* and *Staphylococcus aureus* [26].

We and others have previously appended ATCUN motifs to known AMPs and shown that the new peptides had an enhanced antimicrobial activity [27,28]. The evaluation of the mechanism of action of the resulting conjugates indicated that the increase in activity arises from the added ROS-generating ability of the peptides. In this study, we report the use of two additional ATCUN motifs (Leu-Lys-His, LKH; and Arg-Thr-His, RTH) that were selected from a library of ATCUN peptides for their ability to rapidly produce ROS. We added the sequences to Anoplin (GLLKRIKTLL-NH_2), a peptide with membrane lytic activity. Our results indicate that the ATCUN sequences LKH and RTH can be used to successfully increase the antimicrobial activity of peptides that target membranes.

2. Methods

2.1. Antimicrobial assay

The antimicrobial susceptibility testing was done using a method suggested by Hancock *et al.* [35]. Single colonies of *E. coli* (MG1655, WT), and *Bacillus subtilis* (PS832) were grown to mid-logarithmic phase in Mueller–Hinton Broth (MHB). Then a 50 μL of a twofold serial dilution series of the test peptide were placed in each well of a sterile 96-well polypropylene plate. A 50 μL aliquot of bacterial suspension (10^6 CFU/mL) was added to each well. Ampicillin and Kanamycin were used as positive control. The plates were incubated for 18–20 h at 37 °C, after which, Minimum Inhibitory Concentration (MIC) was defined as lowest concentration that prevented visual growth of the bacteria. *P* value were calculated using One-Way ANOVA function of GraphPad Prism 6.0; statistical significance was set at $P < 0.05$.

2.2. Laser confocal microscopy

Peptides were fluorescently labeled using 5(6)-carboxyfluorescein coupled to the ε-amino group of an additional Lys residue placed between the ATCUN motif and Anoplin. Mid-logarithmic cells were incubated with the FITC-peptides at their MIC (total volume 25 μL). Labeling was allowed to proceed for 15 min; after which, 1 mL of PBS was added to the mixture and the cells were pelleted using a microcentrifuge. The supernatant was removed and 50 μL of sterile PBS was used to resuspend the cells. Then 40 μL of the cell suspension was placed in a poly-L-lysine coated glass slide. The images were acquired using a 100× oil immersion objective mounted on a Nikon A1R Laser Confocal Microscope. The GFP channel was used for detection of probe, and no further image manipulation was done.

2.3. Lipid peroxidation assay

The *E. coli* strain MWF1 (*fabR::kan recD::*Tn10) were obtained from Prof. Charles O. Rock of the University of Tennessee in Memphis and is described in Refs. [29,30]. *E. coli* MWF1 were grown to mid-logarithmic phase and were harvested, washed and resuspended in ROS buffer (20 mM HEPES, 100 mM NaCl, pH 7.40). An aliquot of the cell suspension was incubated with 10 μM Cu^{II}-ATCUN-Anoplin (made by mixing 1.5 eq of ATCUN-Anoplin with 1 eq of Cu^{2+} and incubating for 30 min to completely form the complex), 1 mM sodium ascorbate and 1 mM H_2O_2 for 2 h at 37 °C. After which, the TBA Assay was done using a method suggested by Chirico [31]. A 50 μL BHT (butylated hydroxytoluene) was added to the mixture followed by 1.5 mL of 0.44 M H_3PO_4. This was incubated at room temperature for 10 min. Then, 500 μL of 2-thiobarbituric acid was added and the mixture was heated at 90 °C for 30 min. After the mixture was allowed to cool down, a 60 μL portion was injected in a C_{18} analytical column ran at 65% 50 mM KH_2PO_4 at pH 7.00 and 35% methanol for 10 min. The TBA–MDA product was observed as small peak at around 4.6 min. The activity of the Cu^{II}-ATCUN-Anoplin complexes was normalized against that of free Cu^{2+}, which was assigned 100% activity.

2.4. Circular dichroism studies

The CD spectra were recorded on a Jasco J-710 Spectrometer using purified samples resuspended in 25 mM sodium phosphate buffer (NaPB) pH 7.40, or 50% trifluoroethanol in NaPB. Peptide concentration used was 50 μM, and spectra were recorded from 250 nm to 190 nm in a 1 mm quartz cuvette. Each spectrum was the average of 5 accumulations, and the spectra were recorded twice to ensure consistency (both spectra did not deviate by more than 5%). The mean residue ellipticity, [Θ] was calculated using Eq. (1), where Θ is the measured ellipticity, *l* is path length in cm, *c* is concentration in mM, and *n* is the number of residues in the peptide [36]:

$$[\Theta] = 100 \cdot \Theta / l \cdot c \cdot n \quad (1)$$

The percent alpha helix was calculated using Eq. (2) where $[\Theta]_{222}$ is the mean residue ellipticity calculated at 222 nm [37]:

$$\%\ \alpha\text{helix} = [\Theta]_{222} - 3000/33000 \quad (2)$$

2.5. β-Galactosidase leakage assay

Overnight cultures of *E. coli* transformed with the plasmid pUC19 were subcultured 1:20 into fresh MHB and was grown until $OD_{600} \sim 0.6$. Overexpression of β-galactosidase was induced by addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) and incubation for 1 h. The cells were washed with sterile PBS twice and resuspended in 1× PBS. Peptides at concentration 2× their MIC were mixed with an equal volume of bacterial suspension. The mixture was incubated for 1 h at a shaking incubator, after which, the cells were pelleted and 100 μL of the supernatant was transferred to a clean 96-well microtitre plate. A 50 μL solution of 2.4 mg/mL 2-nitrophenyl-β-D-galactoside (ONPG) in PBS was added (final concentration 0.8 mg/mL) and the color development was allowed to proceed for 1 h at 37 °C in the dark. The absorbance of each well at 405 nm was measured using a standard plate reader.

2.6. Hemolytic assay

Packed human red blood cells (hRBCs) with anticoagulant citrate dextrose (ZenBio Inc.) were washed to exhaustively to remove ghost cells and the anticoagulant. Then a 0.8% (v/v) solution of hRBCs were mixed with an equal volume of the test peptide at 2× their MIC. This mixture was incubated for 1 h at 37 °C, after which the RBCs were pelleted and 100 μL of the supernatant was transferred to a 96-well microtitre plate. The absorbance of leaked hemoglobin was measured at 414 nm using a standard plate reader. PBS and 0.1% Triton X-100 was used negative and positive controls, respectively. The percent Hemolysis was calculated using Eq. (3).

$$\%\ \text{Hemolysis} = \frac{A_{\text{peptide}} - A_{\text{PBS}}}{A_{\text{TritonX100}} - A_{\text{PBS}}} \times 100 \quad (3)$$

Table 1
Minimum Inhibitory Concentration (MIC) of synthesized peptides.

Peptide	Minimum Inhibitory Concentration (MIC), μM	
	B. subtilis	*E. coli*
Anoplin	16	32
LKH-Anoplin	8*	8*
RTH-Anoplin	4*	8*

*$P < 0.05$ when compared to MIC of parental AMP.

3. Results and discussion

3.1. Antimicrobial activity

We have previously identified that copper complexes of the RTH and LKH sequences produce ·OH and other ROS at relatively fast rates as compared to other ATCUN complexes. Out of a library of 13 ATCUN sequences tested, copper complexes of RTH and LKH were the third and fourth fastest ROS-producing compounds, but were an order of magnitude faster than any of the remaining nine sequences [27]. To test whether these ATCUN motifs can be used to increase the activity of AMPs, we added these sequences to Anoplin (GLLKRIKTLL-NH_2). Anoplin, a peptide purified from the venom of a wasp, has membrane lytic activity. The new RTH-Anoplin and LKH-Anoplin peptides were found to be more active than Anoplin against both *B. subtilis* and *E. coli* (Table 1). This lead us to hypothesize that ROS formation played an important role in the activity of the conjugates. We therefore evaluated the degree of oxidative stress brought about by the ATCUN-AMP conjugates.

3.2. Cellular localization of ATCUN-Anoplin peptides

To evaluate whether the addition of the ATCUN motifs RTH and LKH changes the localization of Anoplin, we used laser confocal microscopy to visualize the fluorescently-labeled peptides upon incubation with *E. coli*. The 5(6)-carboxyfluorescein-labeled peptides were synthesized by coupling the fluorescent probe to the ε-amino group of an extra Lys residue placed between the ATCUN motif and Anoplin domains. Similarly to what we have previously observed, the ATCUN-derivatized peptides did not show a change in the localization of the peptide. As observed in Fig. 1, the three peptides localized at the bacterial membrane.

3.3. Lipid peroxidation activity of ATCUN-Anoplin

Since Anoplin has been shown to localize in the bacterial membrane, the ATCUN-Anoplin peptide can form ROS within the membrane and cause oxidation of the fatty acids. To test this hypothesis, we studied the extent of oxidative damage brought about by ATCUN-Anoplin on the bacterial surface. We incubated *E. coli* MWF1 (a strain containing elevated levels of unsaturated fatty acids in its membrane due to a *fabR* mutation, [29,30]) with copper-bound ATCUN-Anoplin (Cu^{II}-ATCUN-Anoplin) and detected the products of lipid oxidation through a standard thiobarbituric acid (TBA) assay [31]. The Cu^{II}-ATCUN-Anoplin complexes were prepared by incubating 1 equivalent of Cu^{2+} with 1.5 equivalents of peptide. As previously reported by Cowan, the slight excess of the peptide is required to avoid the presence of free Cu^{2+} ions that can cause oxidative damage. As can be seen in Fig. 2, Cu^{II}-RTH-Anoplin was more active than the LKH derivative, trailing the trend

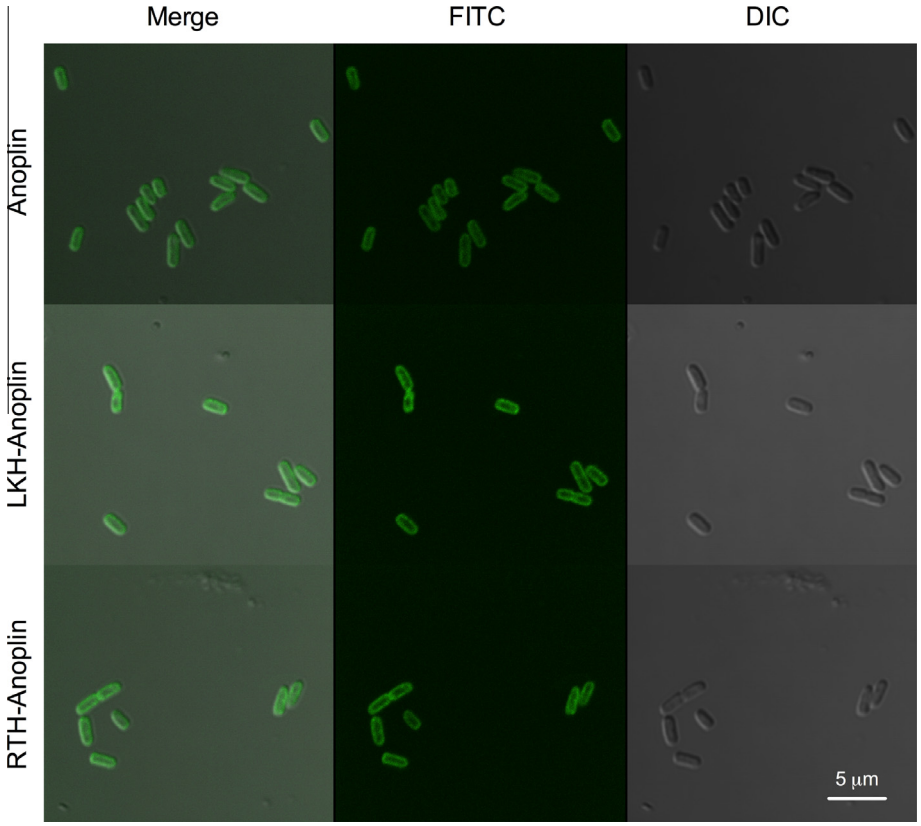

Fig. 1. Laser confocal microscopy fluorescence (middle) and differential interface contrast, DIC (right) images of live *E. coli* cells exposed to 5(6)-carboxyfluorescein-labeled Anoplin (top, 32 μM), LKH-Anoplin (middle, 8 μM), and RTH-Anoplin (bottom, 8 μM).

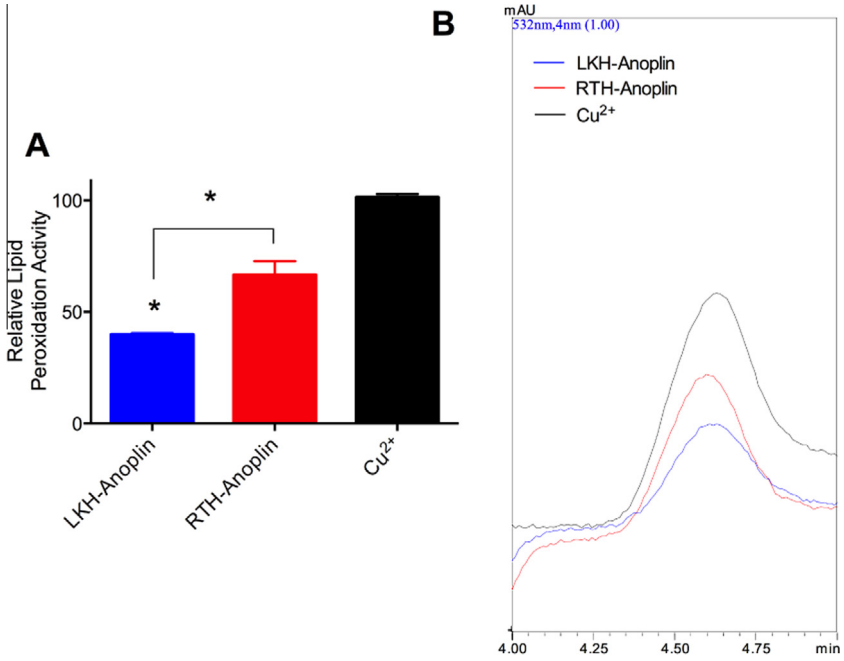

Fig. 2. (A) Normalized lipid peroxidation activity of ATCUN-Anoplin against *E. coli* MWF1. Amount of malonyldialdehyde (MDA) – a product of oxidative lipid damage – was quantified by reaction with 2-thiobarbituric acid (TBA) followed by analytical HPLC separation. Cu^{2+} activity was set to 100% and used as standard for the activity of Cu-ATCUN-AMP complexes. Bars show average of two trials ($^{*}P < 0.05$). (B) HPLC trace of TBA–MDA adduct detected at 532 nm.

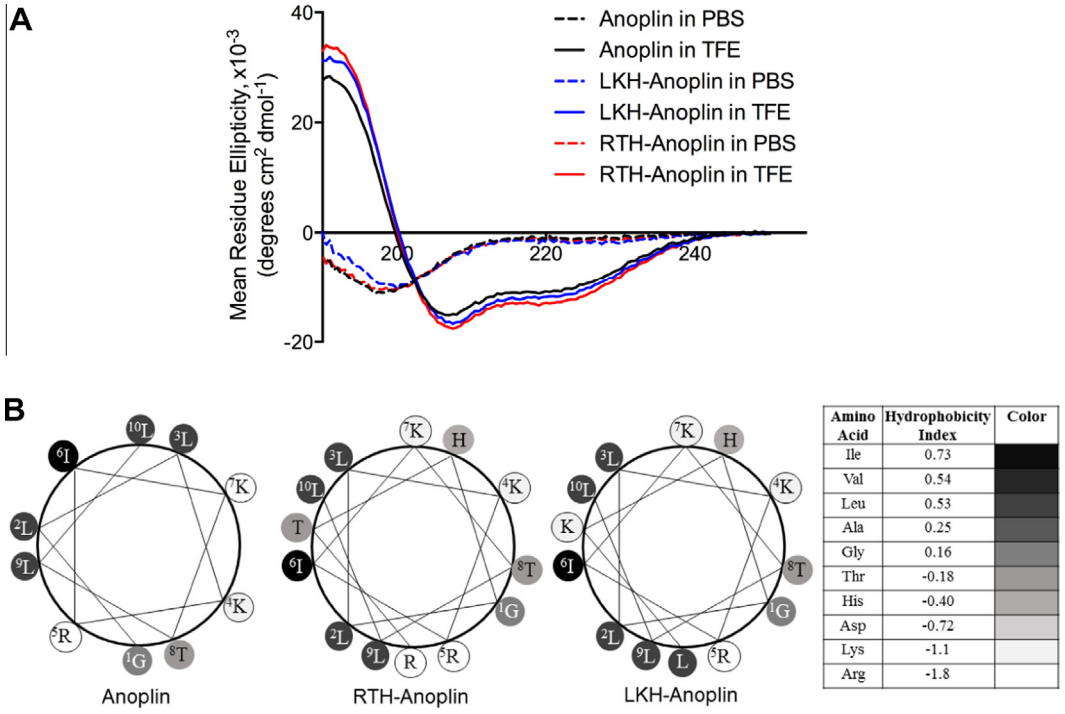

Fig. 3. (A) Circular dichroism spectra of Anoplin and ATCUN-Anoplin in 25 mM phosphate buffer pH 7.40, NaPB (dashed lines) and in 50% TFE in NaPB (solid lines). Spectra show that all peptides adopt an α-helical conformation. (B) Helical wheel diagram of Anoplin and ATCUN-Anoplin peptides. Eisenberg consensus hydrophobicity scale was used [32].

observed in the rates of ROS formation [27]; although both complexes cause less damage than free Cu^{2+} ions.

3.4. Helicity and amphipathicity of ATCUN-Anoplin

We examined the secondary structure adopted by Anoplin and ATCUN-Anoplin using CD spectroscopy with and without the addition of the membrane mimicking agent, 2,2,2-trifluoroethanol (TFE). Fig. 3A shows that Anoplin and ATCUN-Anoplin adopts a random coil conformation in buffer. However, when TFE is added to a final concentration of 50% (v/v), characteristic curves indicative of an α-helix are observed. This suggests that Anoplin and ATCUN-Anoplin adopt a helical conformation when interacting with the bacterial membrane. The resulting curves were used to calculate

the percent α-helical content of each peptide from the observed ellipticity at 222 nm. We found a decrease in the helical content when the ATCUN motif was added to the N-terminal of Anoplin (Table 2). Since protein and peptide function is highly tied to its propensity to adopt a functional secondary structure, we believe that the increased activity of ATCUN-Anoplin is not due to its formation of structured conformations.

Since Anoplin adopts an α-helical conformation, its amphipathicity can be analyzed using a helical wheel diagram (Fig. 3B). An increase in amphipathicity is not expected for the RTH and LKH derivatives, due to the presence of the helix breakers, Lys 2 and Thr 2 in the hydrophobic phase of the helix. This explains why the addition of the ATCUN motifs LKH and RTH resulted in lower percent α helical content for the ATCUN-Anoplin conjugates.

3.5. Membrane permeabilization of ATCUN-Anoplin

Anoplin has been shown to have membrane-permeabilizing activity [33]; therefore, we studied how addition of the three ATCUN amino acids affects Anoplin's ability to disrupt the bacterial membrane. We used the leakage of β-galactosidase to assay membrane permeabilization of *E. coli*. After isolating the leaked β-galactosidase from the bacterial cytoplasm upon exposure to the ATCUN-Anoplin peptides, its activity was monitored by mixing it with 2-nitrophenyl-β-D-galactopyranoside and measuring the increase in absorbance at 405 nm (Fig. 4). The addition of the ATCUN motif did not change the membrane-permeabilizing ability of Anoplin. This result was expected since the overall amphipathicity is not enhanced by the ATCUN motif, resulting in similar membrane disruption activity.

3.6. Hemolytic activity of ATCUN-Anoplin derivatives is as low as that of Anoplin

The synthesized peptides were assayed for hemolysis of human red blood cells. The ATCUN-Anoplin peptides display a hemolytic activity similar to that of the parent peptide (~30%) when tested at their MIC value for *E. coli*, suggesting no changes in Anoplin selectivity upon addition of the ATCUN sequences RTH and LKH.

In summary, the incorporation of the ATCUN sequences RTH and LKH to Anoplin, an antimicrobial peptide with high affinity toward bacterial cell membranes, increases its antimicrobial activity. Our results show that the increase in potency is due to the lipid oxidation ability of the ATCUN motif. It is well known that the consequences of the formation of oxidized lipids range from the formation of hydrophilic pores in the membrane to the promotion of membrane disruption due to extensive oxidative stress, processes that ultimately lead to bacterial death [34]. These results show that addition of an ATCUN motif could be a general approach to improve the antimicrobial activity of membrane-targeting peptides.

Table 2
Calculated percent alpha helix for Anoplin and ATCUN-Anoplin.

Peptide	% α helix	
	25 mM NaPB, pH 7.40	50% TFE in 25 mM NaPB
Anoplin	0	56
LKH-Anoplin	0	45
RTH-Anoplin	0	51

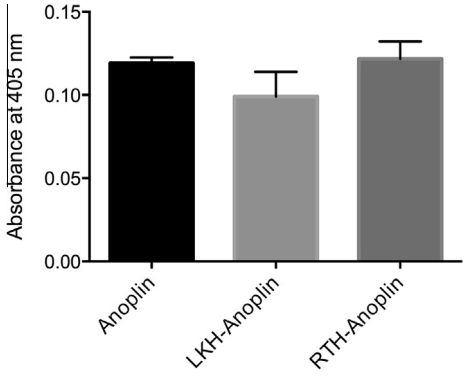

Fig. 4. Membrane lytic activity of ATCUN-Anoplin. Bacterial membrane damage was assessed by quantifying the amount of leaked β-galactosidase upon incubation of *E. coli* with the AMPs. Increase in absorbance of ONPG as a result of β-galactosidase activity was measured for 1 h.

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