Journal of Immunobiology
ISSN 2476-1966
Holthaus et al, J Immuno Biol 2016, 1:1
Research Article
Open Access

Microbicide Activity of Two Reptilian Antimicrobial Peptides on Gram Positive and Gram Negative Bacteria

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Received date: December 10, 2015; **Accepted date:** January 19, 2016; **Published date:** February 4, 2016

Abstract

Previous *in-vivo* studies have isolated and identified peptides with typical molecular anti-microbial characteristics in reptiles. In the present study we have tested the putative antimicrobial action of a lizard cathelicidin and of a turtle beta-defensin using the broth microdilution assay on Gram positive and Gram negative bacteria. The addition of the peptides at concentrations indicatively ranging between 0.05-1.9 mg/ml (cathelicidin) and 0.69-4.14 mg/ml (beta-defensin) inhibited bacterial growth after 3 hours of incubation as determined by their MIC and IC_{50} values. Due to the poor solubility and the medium interference the real concentration of the delivered peptides to the bacterial cultures was uncertain. The qualitative evaluation of the anti-microbial damage after treatment with the peptides was done under the electron microscope that showed some alteration and rupture in the plasma membrane, lowering of the ribosomes, swelling and clumping in nucleoid region of Gram negative (*E. coli*) and Gram positive (*S. aureus*) bacteria. Immunogold labeling against the two peptides indicated that the peptides were localized not only on the plasma membrane and in cytoplasm of the treated bacteria, but also in the nucleoid region and its protein scaffold. The present ultrastructural study suggests that these peptides operate a cellular damage initially on the plasma membrane but further also in the ribosomes and on the DNA or its associated proteins.

Keywords: Reptiles; Antimicrobial peptides; Bacteria; Antimicrobial tests; Ultrastructure.

Introduction

Protection from potentially pathogenic infections from microbes occurs through different mechanisms, including the production of antimicrobial peptides [1]. Numerous antimicrobial peptides responsible for a strong innate immunity have been discovered in prokaryotes and eukaryotes [2-5]. Antimicrobial peptides are composed of 8-60 or more amino acids and include several categories among which the best known include the beta-defensins and cathelicidins [6,7]. The potential utilization of these molecules as effective new antibiotics is of paramount importance in recent times due the mounting resistance of numerous pathogenic microbes to old and new classes of antibiotics, and therefore efforts in discovering effective new drugs is a very active field of modern infective research [8]. Antimicrobial peptides are not a homogeneous class of compounds, but show a broad diversity in structure and antimicrobial spectrum and interactions [9].

Previous studies, based on the observation of the high resistance of lizards and turtle to wounds which showed the presence of numerous intercellular and intracellular bacteria in the epidermis [10-13], suggested that potent antimicrobial peptides were possibly involved in the outstanding immunity present in these reptiles. This hypothesis was later confirmed by the isolation of numerous beta-defensins and some cathelicidins from lizard and turtle [14-16], and from their prevalent localization in granulocytes and activated keratinocytes [13,17]. The association of immunoreactivity for both beta-defensins and cathelicidins with bacteria localized in the stratum corneum further suggested the presence of an antimicrobial barrier in the epidermis, possibly derived from the release of antibacterial molecules that can reach the superficial part of the stratum corneum. Therefore reptiles among amniotes may represent an interesting source of potentially useful peptide antibiotics for medical utilization [8,18,19].

A direct proof of a true antimicrobial affect for the peptides characterized in both turtles and lizards awaits further studies testing the identified molecules on microbial cultures. The present study address the above goal, documenting a cytotoxic effect of two among the most abundant antimicrobial peptides previously characterized in a turtle and a lizard, on cultured bacteria. The antimicrobial effect has been detected using microbial cultures of Gram negative and Gram positive bacteria, and the microbicide action was documented by determining the degree of growth inhibition and evaluating the ultrastructural damage on bacterial cells.

Materials and Methods

Bacterial strains

We used as test organisms Gram negative and Gram positive bacteria represented respectively by Escherichia coli (strain DH5a) and *Staphylococcus aureus* (strain ATCC 2913). The *E. coli* strain was stored in our lab at -80°C and the *S. aureus* strain came from an LB/agar plate. Both strains were cultured freshly for 24 hrs, and shaken at 220 rpm at 37°C in LB Lennox broth for the experiments.

Peptides

Two reptile antimicrobial peptides of 40 amino acids selected by us were synthesized by ProteoGenix Biotec Company, France, as a peptide synthesis service. These cationic antimicrobial peptides were selected on the sequences of a cathelicidine detected in the lizard *Anolis carolinensis* (AcCATH-1, [15]) and of a beta defensin detected in the

turtle *A. spinifera* (TuBD-1, [14]). The amino acid sequences of both antimicrobial peptides are shown in Table 1. Dissolved stock solutions were prepared by the producing Company. In order to avoid that the peptide solvents used in the experiments could also produce damaging effects on our tested microorganisms, the peptide solutions were tested in two different trials for their inhibitory effects. In one experiment the peptides were removed through filtering and in another experiment by reproducing the composition of the solvent and utilizing this solution on the bacterial strains omitting the peptides. The vehicle solution for the turtle beta defensin did not influence bacterial growth at any tested concentration and also the solvent of the lizard cathelicidin did not affect bacterial growth at the employed concentration.

Peptide	Concentration in mg/ml							
	Gram negative *E. coli*				Gram positive *S. aureus*			
	No effect	IC50	MIC least effect	MIC 100%	No effect	IC50	MIC least effect	MIC 100%
TuBD-1 IIGTAICIRRRGACFPIRCPL-YTVRIGRCGLALPCCRWYR	0.5	0.81	0.69	4.14	0.5	1.14	0.69	4.14
AcCATH-1 SLIVVTCDAAVQDDPQMTR-FRGLGHFFKGFGRGFIWGLNH	0.037	0.04	0.062†	*1.90	0.05	0.15†	0.095	*1.90

Table 1: Antimicrobial activity of TuBD-1 and AcCATH-1 against *E. coli and S. aureus* *, at this concentration the solvent likely has also an antimicrobial effect. † value is indicative, because in the upper range of doses there was interference with the solvent. Note: MIC of 100% was defined as inhibiting ≥ 99.9% of bacterial growth.

Media used

Lennox Broth (LB) containing 10 g/l tryptone, 5 g/l yeast extracts and 5 g/l NaCl, was utilized as the standard medium. This medium had a physiological pH and salt concentration of 86 mM. Due to the low solubility of the peptides in the medium and to the possible interactions with salts and organic components we attempted to introduce some variants in order to increase the peptide solubility, like a low salt Medium (10 mM instead of 86 mM as in the original medium), and the addition of 0.01% or 0.025% acetic acid. Another medium utilized was a 0.1 M Tris-HCl buffer solution at pH 6.8, modified with the addition of NaCl to obtain a final concentration of 36.7 mM NaCl.

Antimicrobial assays

Initially the peptides were tested on LB/agar in Petri plates using the colony counting assay but, probably due to the binding of the charged peptides to complex carbohydrates present in the agar [20], no antimicrobial activity was detected. Therefore, the broth micro dilution assay was applied to samples, and the incubation with the peptides was done using LB as described before as a medium. This procedure was followed by plating the surviving bacteria from the test solution on agar in order to determine the antimicrobial activity of the peptides using the colony counting assay.

Prior to testing a subculture of the bacterial strain, the culture was grown at 37°C until the concentration of bacteria reached a mid-logarithmic phase (about 3 hours). After measuring the Optical Density at 600 nm (OD600), the bacterial culture was diluted in the standard medium (LB) to obtain 106 colony-forming units per ml (CFU/ml).

We tested the peptides at concentrations ranging from a minimum of 0.05 µg/ml up to 4.14 mg/ml. Peptides were diluted to the different testing concentrations in 50 µl LB and added to an equal volume of bacterial solution in a 1:1 dilution, and therefore the final bacterial solutions contained 5×10^5 CFU/ml. The final inoculated volumes of 100 µl were then incubated for 3 hrs at 37°C, and shook at 220 rpm. After this period the bacterial solutions were diluted on a 10 fold base, and they were plated in duplicate on Petri dishes (60 mm Ø). After incubation for 18-20 hrs at 37°C in the Petri dishes, the CFU were counted and compared to control cultures grown with no addition of the peptide. The antimicrobial activity was expressed as % of bacterial growth inhibition with respect to the controls, and it was plotted against the tested concentrations of peptides. Using linear regression, the half maximum inhibitory concentration (IC_{50}) was calculated with Excell's ED50V10 add-in method. We also determined the minimal inhibitory concentrations (MIC) at 100% growth inhibition and at the minimum effect on the bacterial cultures. All the results were based on the mean value obtained by at least three independent trials performed in duplicate. The peptides did not show any activity when plated on agar; therefore the colony counting assay was not used for testing the peptides but merely to quantify the growth inhibition obtained by the broth micro dilution assay.

Ultrastructural evaluation of the damage

We sampled controls and tested colonies (2 × 4 mm large) that were growing on the LB-agar substrate (arrowheads and arrows in Figure 1), and that showed different degrees of inhibition related to the peptide used (CATH-1 at 95 µg/ml and TBD-1 at 1.0 mg/ml). Using a sharp razor blade and a tweezer, the colonies of interest grown on the Agar substrate were collected from their Petri dish and immediately fixed. The fixative contained 4% Paraformaldehyde in 0.12 M Phosphate buffer at pH 7.2, and fixation lasted 3 hours at room temperature. After rinsing in the Buffer, the samples were dehydrated in ethanol up to 90% and embedded in Bioacryl Resin under UV at 0-4°C (Scala et al. 1992). Using an ultramicrotome, 1-2 µm thick sections of the samples with their agar support were collected, and the presence of bacteria was systematically checked after staining the sections with 0.5% Toluidine blue. After identifying useful area containing groups of bacteria, thin sections of 40-90 nm were collected on 200-300 mesh Copper or nickel grids for the following study under the transmission electron microscope.

For the routine morphological study, the samples were stained for 30 minutes in 1% uranyl acetate and 5 min in 0.01 M lead citrate, rinsed in water and dried. For ultrastructural immunocytochemistry, two polyclonal rabbit antibodies against AcCATH-1 and TuBD-1 were utilized, as previously specified [12,13]. Briefly, sections on nickel grids were incubated for 3-4 hours at room temperature with the primary antibody at a dilution 1:100 in 0.12 M Tris buffer pH 7.2 containing 1%

Bovine Serum Albumine and 0.01% Triton-X. In control sections, the antibody was omitted in the incubation step. After rinsing in the buffer, the sections were incubated for 1 hour at room temperature with an anti-Rb secondary antibody conjugated with 5 or 10 nm gold particles, rinsed in buffer, in distilled water, and dried. The sections were observed under a Zeiss C10 Transmission Electron Microscope operating at 60 kV, and the images were recorded by a digital camera or photographed with Kodak films (EM Film 4489).

Results

Antimicrobial assays

Both antimicrobial peptides tested showed to negatively influence bacterial growth in *E. coli* and *S. aureus* (Figure 1 and Table 1). One of the problems we encountered in trying to establish a testing protocol was that both peptides did not dissolve well in the medium and this probably diminished their potential activity and availability to the bacterial targets. Another problem was that the solvent of the cathelicin utilized by the producer for the production of the peptide showed inhibitory side effects. Since we could not identify a suitable testing medium where peptides were solubilized efficiently, the calculated concentration in our tests should be considered only indicative.

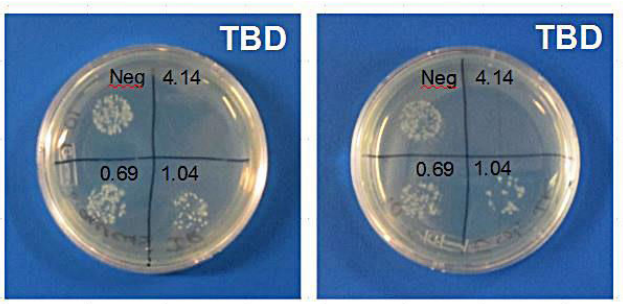

Figure 1: Examples of visible antimicrobial effect on colonies of *E. coli* (left) and *S. aureus* (right) grown in Petri dishes after treatment with TuBD-1 (TBD). The concentrations of the peptides are indicated in mg/ml. Neg is the negative control (untreated, arrowheads) while 4.14 is the MIC (complete inhibition). The other concentrations indicate the least effect (0.69) and an intermediate concentration (1.04). The latter was utilized for the study under the electron microscope (arrows).

As a general result in our tests, although the turtle beta defensin (TuBD-1) did inhibit the growth in both tested bacterial species, its effect was less pronounced compared to the lizard cathelicidin. TuBD-1 in particular did not solubilize well in the employed medium, and form irregular precipitating aggregates. Despite of this drawback it was determined that the IC_{50} for the turtle peptide was indicatively at 0.81 mg/ml for *E. coli* and 1.14 mg/ml for *S. aureus*. There was no sign of inhibition under 0.5 mg/ml of peptide concentration, while the MIC was at 0.69 mg/ml. No growth at all was seen at 4.14 mg/ml of turtle defensin for both bacterial species (Figure 1 and Table 1). Both Gram positive and Gram negative species showed the same MIC for TuBD-1, but *E. coli* was more sensitive, and showed an average inhibition of 78% against 51% inhibition for *S. aureus* with a concentration of 1.04 mg/ml. Also, the IC_{50} of *E. coli* was lower than the IC_{50} for *S. aureus* (Table 1)

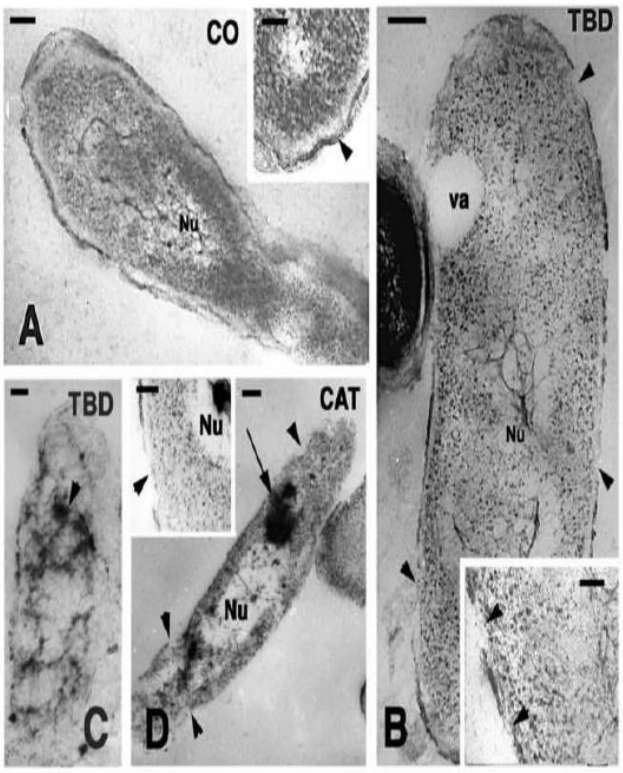

Figure 2: Ultrastructure of normal (A) and damaged (B-D) *E. coli* after TuBD-1 (TBD) and AcCATH-1 (CAT) treatment. A, untreated control cell (CO) showing the central nucleoid region (Nu). Bar: 300 nm. The inset shows the continuity of the cell membrane (arrowhead). Bar: 200 nm. B, damaged bacterial cell after treatment with TuBD-1 (TBD). The cell membrane is discontinuous (arrowheads), the electron-pale cytoplasm is vacuolated (va) and ribosomes are diluted, and the nucleoid (Nu) is not well distinguished from the cytoplasms. Bar: 250 nm. In the inset, the arrowheads point to a discontinuous cell wall and plasma membrane, Bar: 100 nm. C, advance degenerated bacterium after TuBD-1 administration. The arrowhead indicates clumped electron-dense globules while no ribosomes and plasma membrane are present and the cell content directly contacts the extracellular medium. Bar: 100 nm. D, damaged bacterial cell after AcCATH-1 application featuring the enlarged empty nucleoid region (Nu), large electron-dense globules (arrow) and loss of the cell wall and plasma membrane (arrowheads) so that the cytoplasm is exposed. Bar: 100 nm. The inset details on the discontinuity of the cell wall and plasma membrane (arrowhead). Nu, pale nucleoid. Bar: 100 nm.

Also the lizard peptide (AcCATH-1) did not completely dissolve and tended to precipitate, so that the effective concentration available for the anti-microbial effect was lower than the initial concentration. Despite of this drawback, the lizard cathelicidin (AcCATH-1) showed an IC_{50} of 62 µg/ml on *E. coli* (Table 1), but started to inhibit growth at 50 µg/ml and showed no effect at 37 µg/ml. The test using AcCATH-1 on *S. aureus* showed an IC_{50} of 150 µg/ml and the concentration with no inhibitory effect was at 50 µg/ml, therefore higher when compared to that for *E. coli*.

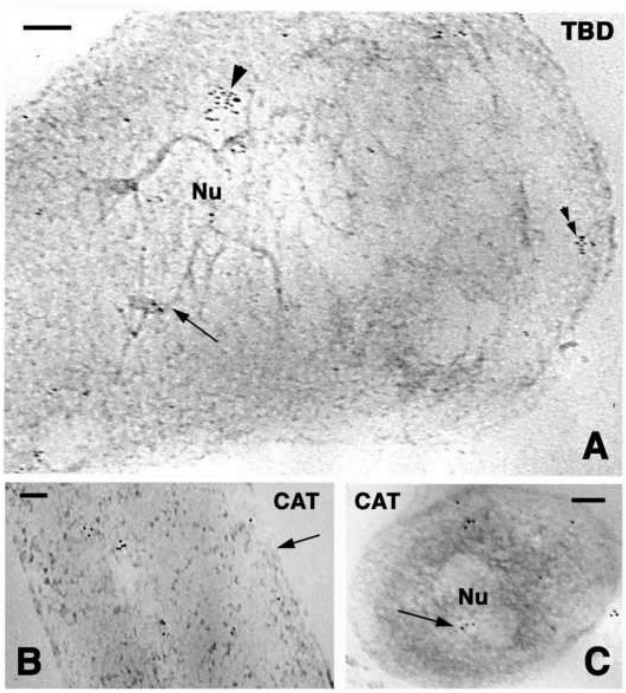

Figure 3: Immunolabeling for TuBD-1 (TBD) and for AcCATH-1 (CAT) in *E. coli* 3 hours after the treatment. A, detail of a cell showing gold particles localized in the peripheral cytoplasm (double arrowhead), nucleoid (arrowhead), and on the protein scaffold (arrow). Bar: 100 nm. B, intracellular labelling in a bacterium with rupture of the plasma membrane (arrow). Bar: 100 nm. C, cross-sectioned bacterium showing labelling in the cytoplasm and Nucleoid (Nu) region (arrow). Bar: 100 nm.

The total inhibition was the same in both species at the concentration of 1.9 mg/ml of cathelicidin but, due to the possible damaging effects of the solvent at this relatively high concentration, these results were discharged. In conclusion, as the results obtained with the turtle beta-defensin, also the lizard cathelicidin showed a stronger inhibitory effect on *E. coli* than on *S. aureus*.

We tried alternative protocols in the attempt to improve the peptides solubility but with no success. When glacial acetic acid at 0.01 and 0.025% was added to the peptide solutions, no effect was elicited aside a negative impact on the growth of bacteria . In another attempt to increase the antimicrobial peptide activity on the bacteria we tested another medium (0.1M Tris HCL) as well as a low salt variant of the LB-medium (10 mM NaCl instead of 86 mM). In the modified 0.1 M Tris HCL buffered medium the turtle beta defensin eventually showed a good solubility, but the buffer alone had a strong inhibitory effect (over 90%) on bacterial growth, which made it unsuitable . The low salt LB variant did not increase the peptide activity but likely influenced bacterial growth, and no further work was carried out following these alternative protocols.

Ultrastructural analysis on *E. coli*

The number of bacteria observed in each thin section analyzed under the electron microscope (12 thin sections in total) ranged between 30 and 60 (*E. coli*). The qualitative observations on untreated *E. coli* showed the typical ultrastructure with numerous free ribosomes surrounding the nucleoid region, and a complete cell wall and plasma membrane surrounding the perimeter of the cell (Figure 2A). Damaged bacteria, with membrane or cytoplasmic alterations, were occasionally seen in untreated cultures.

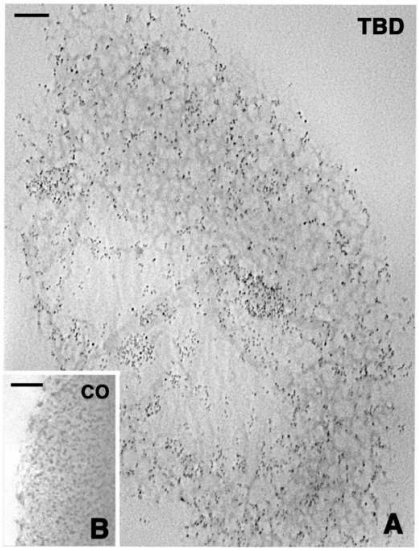

Figure 4: Degenerating immunolabeled *E. coli.* A, Largely degenerated bacterium missing of cell membranes, ribosomes and nucleoid region, and intensely immunolabeled for TuBD-1 (TBD). Bar: 100 nm. B, immunonegative control section. Bar: 200 nm.

The observations on samples after 3 hours of incubation with 1.0 mg/ml of Turtle BD-1, showed that most bacteria (roughly over 80% of recognizable bacteria) appeared damaged in both the cell wall and plasma membrane as well as in the ribosome number (decreased) and in the nucleoid region (Figure 2B). The degree of damage varied from swollen bacterial cells to completely degenerated cells without recognizable cell organelles. In the slightly altered bacteria, the number of ribosomes appeared reduced and the protein scaffold in the nucleoid region appeared irregularly dilated while numerous discontinuities were present along the cell wall (Figure 2B). In other bacteria, cell degeneration was more advanced to the point that not only the cell wall was largely absent but also the cytoplasm appeared devoid of ribosomes while numerous irregular clumps of electron-dense material were present (Figure 2C).

A similar damage over many bacterial cells (roughly over 80% of recognizable bacteria, but likely clumped material derived from completely destroyed bacteria was also present in the sections) was also detected after treatment with 95 μg/ml of the lizard cathelicidin (Ac-CATH-1). The damaged bacteria after 3 hours of peptide incubation appeared generally in a very advanced stage of degeneration, featuring numerous discontinuities along the cell wall and plasma membrane, strong reduction of ribosomes, appearance of flocculent material in the cytoplasm and of dense roundish clumps of material often associated to the nucleoid (Figure 2D). The nucleoid region in particular was swollen and scarce protein scaffolds were seen.

The immunogold observations on damaged but still recognizable bacterial cells of *E. coli* showed the presence of gold particles over the cytoplasm and the nucleoid area using both the turtle beta-defensin and lizard cathelicidin, including the protein scaffold of the nucleoid (Figure 3). This observation indicated a complete penetration of the

peptides in all regions of the bacterial cells. In some residual bodies resulting from advanced stages of bacterial cell degeneration, the immunolabeling was seen over most of the bacterial remnants where a nucleoid and cytoplasmic regions were no longer detectable (Figure 4A). No labeling was seen in control sections (Figure 4B).

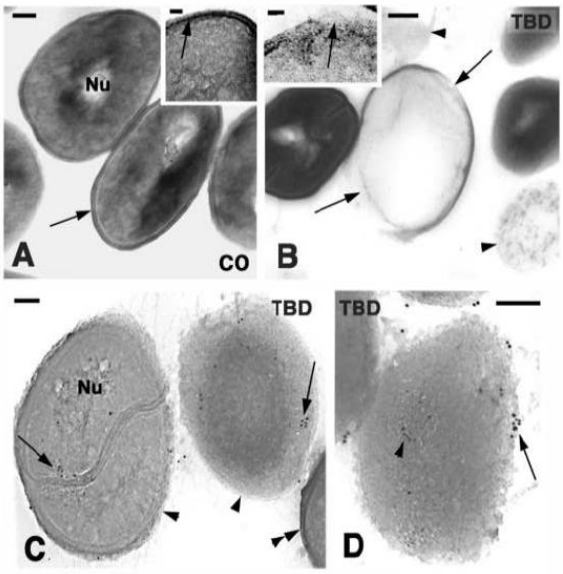

Figure 5: Structure (A,B) and immunogold labeling (B,C) of *S. aureus* cells treated with TuBD-1 (TBD). A, untreated samples (CO, control) showing a dense cytoplasm, a complete cell wall (arrow) and centered nucleoid region (Nu). Bar: 100 nm. The inset shows the continuity of the cell wall (arrow). Bar: 50 nm. B, after 3 hour of treatment with among normal cells (darker) degenerated and electron-pale cells with coagulated cytoplasm devoid of ribosomes are visible (arrowheads). The arrows point to discontinuities on the cell wall of a ghost cell. Bar: 100 nm. The inset details the discontinuity of the cell wall (arrow). Bar: 50 nm. C, Two treated cells (Nu, nucleoid), one in division (left), show labeling in the peripheral cytoplasm and along the cell wall (arrows), the latter largely missing (arrowheads, compare with the cell wall in the nearby cell, double arrowhead). Bar: 100 nm. D, other treated and degenerating protoplast showing cluster labeling (arrow) along the irregular cell periphery while gold particles are also present in the cytoplasm (arrowhead). Bar: 100 nm.

Ultrastructural analysis on *S. aureus*

The number of bacteria observed in each thin section analysed under the electron microscope (12 thin sections in total) ranged between 150-200 in *S. aureus*. In the untreated cultures most of the cells were intact and typically surrounded by a thick cell wall (Figure 5A), and few protoplasts (cells without the cell wall) but rare degenerated cells were present. In the treated culture at 95 μg/ml of Ac-CATH1, a clearly visible damage on the cell morphology interested a higher number of bacteria (roughly 30-40%) that in normal controls.

The cellular alteration varied from the disappearance of the cell wall in numerous bacterial cells that gave rise to more frequently detected protoplasts, to a cytoplasmic coagulation within the damaged protoplasts or, in other cases, to the formation of ghost cells devoid of cytoplasm content and the rupture of the cell wall and plasma membrane. The observation of the immunolabeling detected under the electron microscope, aiming to evaluate the penetration and localization of the turtle beta-defensin (TuBD-1) in the treated cells of *S. aureus*, showed that the gold particles were mainly distributed on the peripheral areas of the bacterial cells and along the cell wall (Figure 5B and 5C). Also the central cytoplasm of damaged cells and the plasma membrane of protoplasts were immunolabeled. Often the gold particles formed clusters, especially along the damaged cell wall and the plasma membrane that appeared frequently discontinuous (Figures 5C and 5D). Although observed less frequently, also the nucleoid region was immunolabeled for the turtle beta-defensin. No labelling was seen in control sections.

Discussion

Antimicrobial assays

The protocols established for testing anti-microbial peptides may give un-accurate results due to a variety of conditions such as poor solubility of the peptides, medium interactions, pH, ionic strength and salt concentration, all factors that can influence the effectiveness on the tested bacterial strains. Furthermore a medium should mimic the in vivo environmental conditions of the organism from which the peptide was originated to assure a realistic functional test, but this was not possible in our case. Various studies have analysed these interactions [21-25], but the mechanism of peptide availability to bacteria in culture has not been fully elucidated.

In our attempts to test some antimicrobial activity of our peptides, different problems arose in order to obtain a realistic MIC value that could actually correspond to the effective MIC of the condition in vivo. One problem is related to the right folding of the peptides utilized in our test since it is known that antimicrobial peptides must have a specific three-dimensional form (the effective folded peptide) in order to exert their anti-microbial effect [2,26]. Peptides without the right folding can have very little to no antimicrobial effect at all. In the present study we could not determine the concentration of the effective folded peptides within the available mix of peptides provided by the Peptide Synthetic Company, therefore the reported concentrations are only indicative and the real MIC is likely much lower. Another problem, which was mentioned above, is the poor solubility of the peptides that probably diminished the effective peptide availability in solution compared to the calculated inhibithory concentrations (Table 1).

In order to improve the antimicrobial activity of our peptides we tested some LB variants, but without success since the changes introduced influenced themselves the growth of bacterial and made it impossible to compare the results. Although the Tris/HCl medium gave similar results as in a previous study [20], negative controls showed that the medium alone caused over 90% inhibition, and therefore we could not consider this medium. Despite these problems, the qualitative results clearly showed that a sensible number of bacteria (30-80% or higher) were affected by the peptide solutions, the basis for further more quantitative pharmacological studies. In future studies, the solubility problems should be overcome if these antimicrobial peptides of reptilian origin will be tested in vivo for possible medical applications.

It is believed that antimicrobial peptide characteristics like their net charge and hydrophobicity determine their functionality. Changes in their net charge and hydrophobic ratio can influence both their antimicrobial activity and selectivity [27]. Both our peptides are cationic although their net charge is different, +8 for TuBD-1 and +1 for AcCATH-1, and the index of hydropaty is fairly low for AcCATH-1

(+0.077) when compared to TuBD-1 (+0.463). The lizard cathelicidin showed a MIC over 10 folds lowers than that the MIC of the turtle beta defensin (0.05 against 0.69 mg/ml). Also the MIC value for the total inhibition was 2 fold smaller for AcCATH-1 with respect to TuBD-1. In our testing conditions the lizard cathelicidin (AcCATH-1) that presents a moderate net charge and hydrophobicity appears to function more efficiently than the turtle beta defensin (TuBD-1). Furthermore, *E. coli* strains seem to be more sensitive to both peptides with respect to those of *S. aureus*, perhaps due to the presence of the thicker cell wall in the latter, G+ bacteria. The charge of the tested peptides resembles that of other antimicrobial peptides that in physiological conditions are generally cationic and that assume a secondary amphipathic structure in a hydrophobic environment or when encountering a cell membrane. The conformation of an amphipathic structure seems to be essential, since it forms an alpha helix which lipophilic face allows the solubilization of the peptide when it contacts the phosholipids of the bacterial membrane [28-30]. The initial target of cationic peptides is the anionic bacterial cell membrane where the positively charged peptide binds to the negatively charged phospholipids [31]. No specific receptors are involved in the binding, and this makes difficult for bacteria to develop resistance to these molecules.

In comparison to previous microbiological tests using reptilian antimicrobial peptides, the microbicide concentrations of AcCATH-1 and TuBD-1 peptides appear much higher in the conditions of our experiments mentioned above. In fact, TuBD-1 featured an inhibitory activity from 690 μg/ml up while the lower values for AcCATH-1 was at 37-69 μg/ml. Similar inhibitory concentration to those for AcCATH-1 were obtained using an antimicrobial peptide derived from snake venom (120-130 g/ml for *E. coli*, over 200-250 μg/ml for *S. aureus*; [32]). However a cathelicidin isolated from the snake Bungarus fasciatus was reported to express very low MIC values (0.6-2.3 μg/ml for *E. coli*, and 4.7 μg/ml for *S. aureus*), but for some *S. aureus* strains >100 μg/ml of peptide were needed [33]. A turtle beta-defensin (from Emys orbicularis) also showed very low MIC values, 0.65 and 5.6 μmol/L for respectively *E. coli* and *S. aureus* [34]. Another beta-defensin from the turtle Caretta caretta showed IC_{50} values of 3.3 μM for *E. coli* and 5.1 μM for *S. aureus* [35]. Finally crocodilian antimicrobial peptides (leucrocins) showed very different MIC values from as low as 0.66 up to >156 μg/ml for Staphylococcus sp. Not only the various leucrocins had a variable impact on different bacterial strains but also the bacterial strains showed varying sensitivity to the peptides [36]. These results suggest obvious differences in sensitivity among bacterial species.

Despite the IC_{50} and MIC values for AcCATH-1 and TuBD-1 peptides are apparently higher than other reptilian peptides, their morphological effect on the bacteria seen under the electronic microscope was however impressive.

Morphological alterations

The present ultrastructural study shows that the lizard Ac-CATH-1 and the turtle Tu-BD-1 peptides determine some inhibition of microbial growth that derives from the cell damage to both *E. coli* and *S. aureus* strains. After only 3 hours from the treatment, the ultrastructural analysis has clearly shown signs indicating that both the cells of *E. coli* and *S. aureus* strains are damaged at various degrees. The damage on bacterial cells was variable, often advanced, and numerous aggregates of clumped amorphous masses, often labeled with immunogold likely representing residual bacterial cells, were observed. These uncertain remnants of degenerated bacteria are also a problem in our attempt to give a quantitative esteem of the damage, another reason that makes quantitative determination of the damage very difficult in this study. Therefore in the present qualitative study the main goal was to document the degree of damage in bacterial cells (plain ultrastructure) and the penetration of the peptide inside bacterial cells (immunogold labeling).

We have not determined the effects of the peptides in strains of bacteria treated for longer periods (24 hours is a standard period for traditional antibiotics) but they would have likely been much more dramatic than those here observed after few hours from the treatment. In previous morphological studies on the damage elicited by the treatment with antimicrobial peptides on different strains of bacteria, clear signs of cytological alterations in bacterial cells were seen at 30 minutes, 1, 2, 6, 12, and 21 hours after the incubation with the different AMPs tested [19,37,38].

From the present observations it appears that the cytolytic effects are directly or un-directly elicited not only on the cell wall and the plasma membrane, possibly the primary or however the initial targets of the peptides, but also the ribosomes and the nucleoid region appear subjected to some effect of the peptides. Furthermore, the immunolocalization of the peptides within bacterial cells indicates that after the peptides have penetrated and crossed the cell wall and the plasma membrane, they localize in the nucleoid, suggesting a possible interaction with the bacterial DNA. Previous studies in the vast literature on the sites of action of AMPs have indicated that not only the plasma membrane but also the DNA, RNA and indirectly also protein synthesis can be the targets of some peptides [4,39].

Different models on possible antimicrobial mechanisms for entering the bacterial cell and act on intracellular targets have been proposed [26,40-42]. Once inside the cell the peptides may interact with RNA, DNA and protein synthesis causing their inhibition, and from our observations with immunogold labelling a possible interaction with the DNA of the bacteria is suggested.

Previous studies on the antimicrobial effect from snake peptides that were examined under the electron microscope [38] using peptide concentrations varying from 4-10 μg/ml have shown similar cytolitic effects on bacterial cells as the damages shown in the present study. The damage initially included blebbing of the plasma membranes, the rupture of the membranes with loss of cytoplasmic content, and later the clarification of the nucleoid region while ribosomes disappeared [37]. The deterioration of the bacterial cell structure later leads to the formation of ghost cells that feature a discontinuous cell wall and cell membrane, an extracted content in the cytoplasm or the presence of sparse clumped material without ribosomes, damages frequently observed in our material (Figsure 2 and 4). Similar ultrastructural degenerative aspects were also observed using another beta-defensin peptide, pelovaterin, derived from a soft shelled turtle on Gram+ *Pseudomonas sp* [18] or a snake cathelicidin on Gram- *E. coli* [19], but at a much lower dosage than in our study (12 μg/ml).

In conclusion, these data further indicate that antimicrobial peptides produced in reptiles may represent potential pharmacological drugs after a further trial of pre-clinical tests once their solubilization will be improved [8]. It has been indicated that reptiles have a very efficient innate immunity in part based on anti-microbial peptides since their acquire immunitary system is relatively slow and not as efficient as that of mammals [43]. Particularly in lizards the presence of effective peptides may be linked to the relatively low inflammatory response after wounding, a process that favors the following re-epitelialization and tissue regeneration [10], while in turtle the anti-microbial barrier impedes microbe invasion in the skin [13]. Based on this hypothesis the present explorative study has shown that a lizard

cathelicidin and a turtle beta-defensin are bacterial killers, but their potential as anti-infective agents has to be fully evaluated in further and more specifically designed microbiological and pharmacological studies.

Acknowledgments

The microscopic study was financed through the Comparative Histolab and a 2014 RFO from the University of Bologna. Dr. Sandra Turroni (Department of Pharmacy and Biotechnology, University of Bologna) supplied the *S. aureus* strains for testing. Drs. Alessio Papi and Francesca Borsetti of the Department of Bigea kindly advised in some laboratory operations.

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