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--Manuscript Draft--

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[3] Istituto Zooprofilattico Sperimentale della Sicilia, A. Mirri. Via Marinuzzi 3, 90129 Palermo, Italy.

***corresponding author: domenico.schillaci@unipa.it**

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Introduction

Conventional antibiotics strike targets associated with cell growth and bacteria life resulting in a strong selective pressure for drug resistant microorganisms (Chambers and Deleo 2009). The multidrug-resistance of common Gram-positive and Gram-negative pathogens is a global emergence and new approaches to find new antimicrobials with chemical characteristics different from current antibiotics and hopefully with different mechanism of action, are needed.

Agents targeting bacterial virulence factors without interfering on bacterial viability have the advantage to disarm pathogens without killing them, with a consequent lower pressure in the rise of antibiotic resistant strains (Cascioferro et al. 2015; Cascioferro et al. 2014b). The use of antimicrobial peptides (AMPs) to control infections impair the development of drug resistance due to the multiplicity of their biological activities (Parachin and Franco 2014). AMPs have a high specificity for prokaryotes and a low toxicity for eukaryotic cells and they can interact with the infected host, stimulating his own immune defenses.

The control of bacterial infections becomes very challenging if pathogenic bacteria are organised as biofilm community. Conventional antibiotics effective against planktonic (free living) bacterial cells, might fail in controlling the pathogens organized in community since microbial biofilms are intrinsically resistant to antibiotics (Gilbert et al. 2002). The biofilm is a three-dimensional community of microorganisms embedded in a polymeric matrix, growing attached on biological or artificial surfaces. Biofilm associated infections might be responsible for the rejection of orthopaedic prostheses and other medical devices (Costerton et al. 1999). Biofilms of staphylococcal strains and *Pseudomonas aeruginosa,* in particular are often related to significant delays in wound healing (Metcalf and Bowler 2014).

The search for alternative strategies to conventional antibiotics is particularly desirable for a more efficacious anti-biofilm treatment. We recently reported that the 5-kDa peptide fraction from the coelomocyte cytosol (5-CC) of the *Paracentrotus lividus*, the sea-urchin from Mediterranean sea

showed antimicrobial and anti-biofilm activity against human common pathogens (Schillaci et al. 2010a; Schillaci et al. 2014).

In the 5-CC fraction we found three principal peptides with molecular weights respectively of 1251.7, 2088.1, and 2292.2 Dalton. These peptides correspond to the fragments (9-19), (12-31), (24-41) of β-thymosin of *P. lividus*. The smallest peptide, Paracentrin 1 (SP1), constituted by 11 amino acids, EVASFDKSKLK, was particularly interesting because it showed the chemical-physical characteristics of an antimicrobial peptide (Wang et al. 2009).

The present study was aimed to compare the molecular structures, dynamics, antimicrobial and anti-biofilm activity of the fragment 9-19 of human β4 thymosin ($T\beta4_{9-19}$), EIEKFDKSKLK with the previously described analogue from sea urchin SP1. Human T β4 is considered the principal intracellular G-actin sequestering peptide (Safer et al. 1990) and a human defence peptide in saliva, ocular environment and platelets (Badamchian et al. 2007; Kaur and Mutus 2012; Rossetti et al. 2013; Sosne et al. 2012). It is interesting to highlight that other fragments of human Tβ4 are involved in numerous biological effects *in vivo*: i) the amino-terminal fragment of 4 amino acids (Ac-SDKP) has an anti-inflammatory activity and decreases fibrosis; ii) an amino terminus fragment of 15 amino acids, including Ac-SDKP, interferes with apoptosis and stimulates cell survival; iii) a short peptide of 7 amino acids (aa 17-23), LKKTETQ, in the central actin-binding domain, exerts an angiogenic action and it is effective on wound healing. It has been observed that the intact human Tβ4 showed antimicrobial activity, but no fragment responsible of this action has been identified so far (Goldstein 2007).

In the last years, molecular dynamics (MD) simulations have been routinely performed in order to investigate the interaction between AMP and lipids with an atomistic resolution (Bocchinfuso et al. 2011; Dunkin et al. 2010; Wang et al. 2012). To mimic the protein/membrane interaction, MD simulations can start with a preformed bilayer and a folded peptide. However, in these conditions, it is difficult to reach the equilibrium configuration in short simulation time, due to the slow

molecular diffusion. A more efficient protocol was proposed in 2007 by Esteban-Martín and Salgado (2007), by which unordered lipids are allowed to self-organize in the presence of AMP. This strategy has recently led to successful results (Farrotti et al. 2015; Khatami et al. 2014; Wang et al. 2013)

In the present study we report on MD simulations of the membrane interaction of human Tβ4$_{9\text{-}19}$, which shows a little difference with SP1 from *P. lividus* in some residues near N-terminus. In detail, two bilayer structures have been considered: POPC (1-Palmitoyl-2-oleoylphosphatidylcholine) and POPC:POPG (1-Palmitoyl-2-oleoylphosphatidylglycerol) (2:1), as mammalian and bacterial membrane models, respectively. Additionally the antibacterial and antibiofilm activities against a group of staphylococcal reference strains and *P. aeruginos*a ATCC 15442 are reported.

Materials and methods

Synthetic peptide

The fragment 9-19 of human thymosin Tβ4 was custom synthesized by GenScript, using the specified peptide EIEKFDKSKLK identified by ESI-MS analysis. Fmoc solid phase technology was used to obtain the peptide. The purity (95%) was determined by high performance liquid chromatography (HPLC) and mass spectrometry (MS) analysis.

Molecular Dynamics simulations

MD simulations were carried out in order to establish the molecular folding of the Tβ4$_{9\text{-}19}$ in physiological conditions in silico by using the following protocols (Lentini et al. 2014). In details, 1 μs of MD simulation of the sequence EIEKFDKSKLK was performed at 300 K. The time step was set to 2 fs, and all covalent bonds were constrained with the LINCS algorithm. The Amber99SB-ILDN force field (Lindorff- Larsen et al. 2010) implemented in the GROMACS 4.6.5 software package (Pronk et al. 2013) was used. A triclinic box was added to a depth 1.0 nm on each side of the peptide and it was filled with TIP3P water molecules and 150 mM Na^+ and $Cl^−$ counterions.

The particle mesh Ewald method (PME) (Darden et al. 1993) was used to describe the long-range electrostatics interactions. Energy minimization was run for 5000 steps using the steepest descend algorithm. In a 500 ps equilibration the peptide was harmonically restrained with a force constant of 1000 kJ mol^{-1} nm^{-2} at 300 K, which was gradually lowered until no restrains were applied.

The software packmol (Martínez et al. 2009) was used to generate starting configurations for the study of the interaction of $T\beta4_{9\text{-}19}$ with the two membrane models. The peptide was placed in the centre of a 100 Å cubic box; 128 POPC (for the mammalian model), 86 POPC and 42 POPG (for the bacterial model) and 7500 water molecules, were added in the box. Amber99SB-ILDN was used in combination with the Slipids (Stockholm lipids) force field for lipids (Jämbeck and Lyubartsev 2012a; Jämbeck and Lyubartsev 2012b).

Pressure coupling was applied anisotropically, using a Parrinello-Rahman barostat with a reference value of 1 bar. A short 100 ps equilibration was performed to achieve a realistic density of the system followed by 200 ns production runs. Pictures and Ramachandran plot were obtained by the VMD software (Humphrey et al. 1996). Density profiles were calculated with the GROMACS tool *g_density*. Clustering analysis was performed by *g_cluster* tool, also included in the GROMACS package.

Bacterial strains

We used the following staphylococcal reference strains: *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538, and *Staphylococcus epidermidis* RP62A. We also evaluated the antibacterial activity against *Pseudomonas aeruginosa* ATCC 15442, considered a reference strain in official tests for antibacterial evaluation in vitro (UNI EN European Standard).

Minimum inhibitory concentrations (MIC)

MICs against planktonic strains were evaluated by a micro-method previously reported (Schillaci et al. 2008). Briefly, a series of solution in Tryptic Soy Broth (TSB) with concentrations ranging from

25 to 0.3 mg/ml were obtained by twofold serial dilution in 96 well plate. To each well 10 µl of a bacterial suspension obtained from a 24 h culture which contained 10^6 cfu /ml was added in 100 µl of TSB medium. The plate was incubated at 37°C for 24 h. After this time, MIC values were evaluated by a microplate reader (ELX 800, Bio-Tek Instruments) as the lowest concentration of compound at which the optical density (OD) at 570 nm of the well was comparable to the negative control well (broth only). The activity of human cathelicidin LL-37 (Sigma) and bovine lactoferrin (Sigma) were tested for comparative and quality control purposes.

Evaluation of Biofilm formation and Biofilm prevention assay

The ability to form biofilms of *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 15442 were determined. The procedure, previously described (Raimondi et al. 2012; Schillaci et al. 2010b), was also used to test the activity in preventing biofilm formation of $T\beta4_{9\text{-}19}$, at sub-MIC concentrations of 1.5, 0.7 and 0.3 mg/ml. Briefly, bacteria were grown in Tryptic Soy Broth (TSB, Sigma) which contained 2% glucose overnight at 37°C in a shaking bath and then diluted 1:200 to a suspension with optical density (OD) of about 0.040 at 570 nm. Polystyrene 24-well tissue culture plates were filled with 2 ml of diluted bacterial suspension and different concentrations of the peptide and incubated for 24 h at 37°C. Then, the wells were washed three times with 1 ml of sterile phosphate-buffered saline (PBS) and stained with a crystal-violet solution 0.1% w/v in water. The excess stain was removed by placing the plates under running tap water. Crystal-violet stained adherent bacteria in each well were re-dissolved to homogeneity in 1 ml of ethanol, and the OD was read at 570 nm. Comparing the average of OD the growth control (not treated) wells with that of sample, we calculated the inhibition percentage for each concentration of the peptide by the following formula:

$$\%\ of\ Inhibition = \frac{OD\ growth\ control - OD\ sample}{OD\ growth\ control}\ x\ 100\%$$

Each assay was performed in triplicate and repeated at least twice.

Results

Molecular Dynamics of fragment 9-19 of human Tβ 4

Tβ4:actin complexes were recently crystallized, as reported in, e.g., pdb ID 4PL8 (Xue et al. 2014)]. However, in the present work we have focused our attention on the fragment 9-19 of human Tβ4. The conformation adopted in solution from this small fragment is certainly different from the one adopted by Tβ4. Thus, in order to obtain possible stable folded conformations we have performed 1 μs of MD simulation, during which the undecapeptide EIEKFDKSKLK explored a wide number of backbone conformations as shown by the root mean square deviation (RMSD) in Fig.1A. Interestingly, in this graph there are two time ranges in which the RMSD is constant, between about 550-700 ns and 820-880 ns, respectively, witnessing the presence of two stable conformations in the two time intervals, named B and C, whose shapes in blue strings are also sketched in Fig. 1B and 1C. The reported structures (B and C) were identified utilizing a clustering analysis. A comparison between the conformations B and C and the one extracted from 4LP8 showed, as expected, remarkable differences.

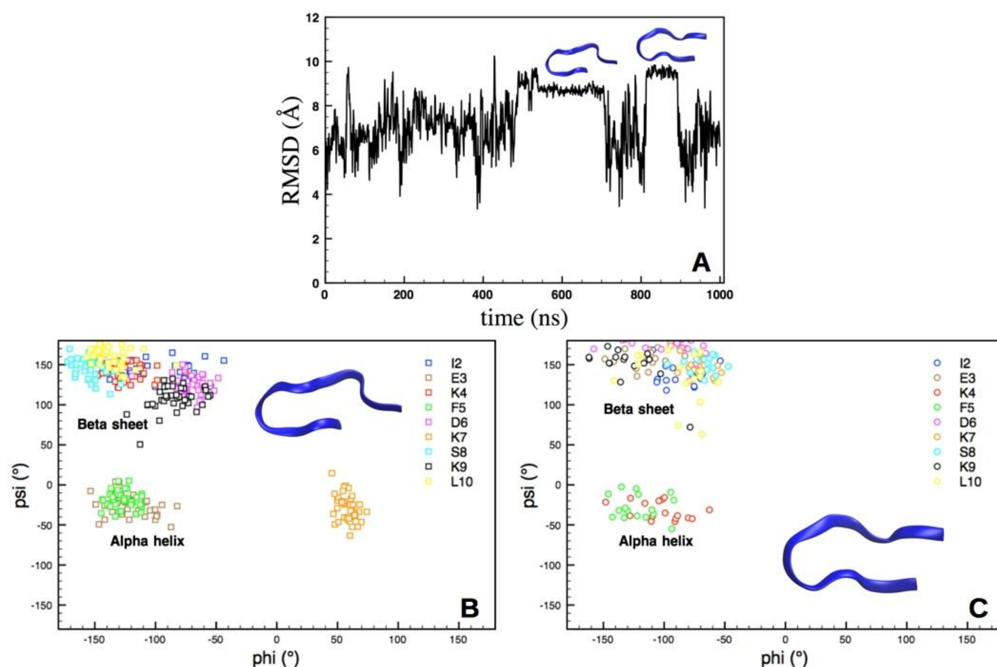


Fig. 1 Plot of the RMSD obtained for Tβ4$_{9\text{-}19}$ up to 1 μs of MD simulation (A) and Ramachandran plots showing the values of psi and phi angles assumed by residues 2-10 of Tβ4$_{9\text{-}19}$ in the two stable conformations (B) and (C).

To analyse these two structures in detail, we have also reported their Ramachandran plots in in Figs. 1B and 1C, obtained after sampling snapshots every 5 ns along the simulation in the above mentioned two time ranges. These plots showed that in this time range the different residues assume preferential local conformations. The most populated conformation is the beta sheet in both structures B and C. Only two residues are in alpha helix conformation in each of the two structures, in particular, residues E^3 and F^5 in the first structure and K^4 and F^5 in the second structure. On the other hand, residue K^7 is in a random coil conformation in the first stable conformation, due to the position of this amino acid in the centre of the loop. Similar results were recently obtained with the analogue fragment, SP1, from sea-urchin (Schillaci et al. 2014).

To compare the structural properties of Tβ4$_{9\text{-}19}$ and SP1, and in particular its effect on their molecular polarity, the most recurrent conformations are reported in Fig.2. Such pictures show that both conformations of Tβ4 are constituted by a central hydrophobic core and by the presence of

peripheral charged residues. On the other hand, the most stable conformation of SP1 possesses a hydrophobic non-amphipathic region placed between E^1 and D^6 constituted by residues V^2, A^3, S^4, F^5. This remarkable difference is a result of the substitution of V^2, A^3, S^4 of SP1 (Fig. 2A) with I^2, E^3, K^4 in Tβ4 (Fig. 2B and 2C).

Concerning the polarity of B and C**,** Fig. 2 shows that both structures are constituted by a central hydrophobic core and by the presence of peripheral charged residues.

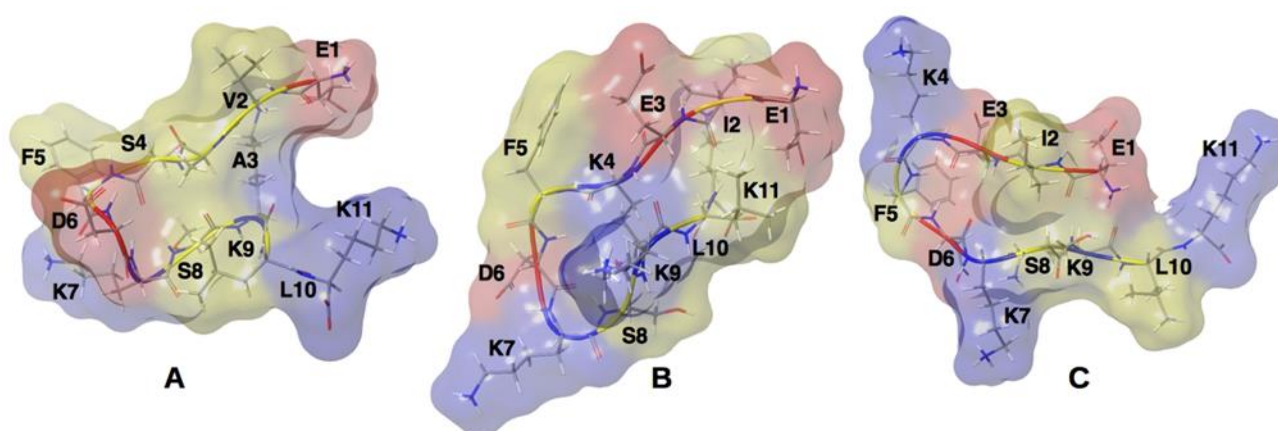


Fig. 2 Molecular representation of the most stable conformations of SP1 (Schillaci et al. 2014) (A) and of Tβ4 (B and C). Color code: acidic residues in red, basic residues in blue, other residues in yellow.

Interaction with model membranes

MD simulations were also performed in order to study the interaction of $T\beta4_{9\text{-}19}$ with the two membrane models: POPC and POPG(2:1). Structure B was used as a starting structure in both simulations. As reported in the literature protocol, (Esteban-Martín and Salgado 2007) lipids are indeed able to self-organize to form a bilayer in a reasonable time scale. As an example, a POPC bilayer without membrane defect is formed after about 50 ns (Fig. 3A). The POPC:POPG bilayer, due to the presence of mixed lipid, requires around 100 ns to assemble (Fig. 3B). During this process, $T\beta4_{9\text{-}19}$ interacts mostly with lipid polar heads, as a consequence of the peptide's polar nature, due to the presence of the several peripheral charged residues.

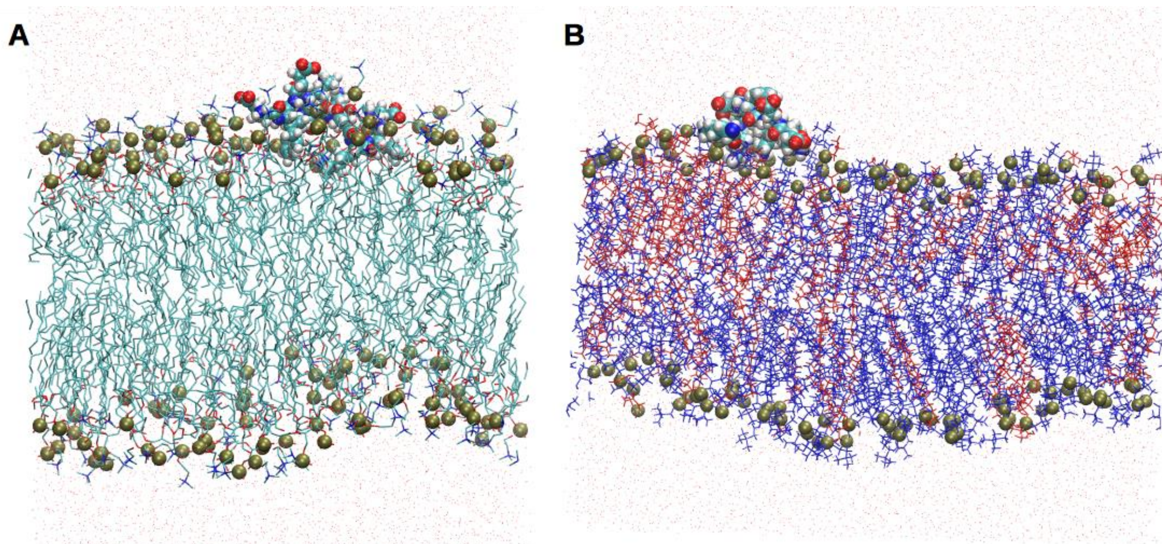


Fig. 3 Two snapshots taken after 100 ns of simulation showing Tβ4$_{9-19}$ interacting with A) the POPC and B) the POPC (blue): POPG (red) models.

The position of the peptide and the lipids evolution during the self-assembly process is summarized in Fig. 4, by density profiles taken at different times of the MD simulations. At the beginning of the simulations water and lipid are mixed and Tβ4$_{9-19}$ is not localized. In the middle of the simulation the POPC bilayer is formed and Tβ4$_{9-19}$ interacts with polar lipid heads until the end of the simulation (Fig. 4B-C top). The POPC:POPG case is less straightforward: first, at ca. 100 ns, the bilayer is formed but water is not completely excluded from the centre. Moreover, the bilayer is not symmetrical, showing slight separation of POPC and POPG in the two leaflets. Tβ4$_{9-19}$ is initially associated with the POPG rich leaflet (Fig. 4B bottom), but in the last part of the simulation Tβ4$_{9-19}$ binds the other leaflet, crossing the box's periodic boundary conditions. Interestingly, such result indicates a binding preference of the peptide for the monolayer with less POPG.

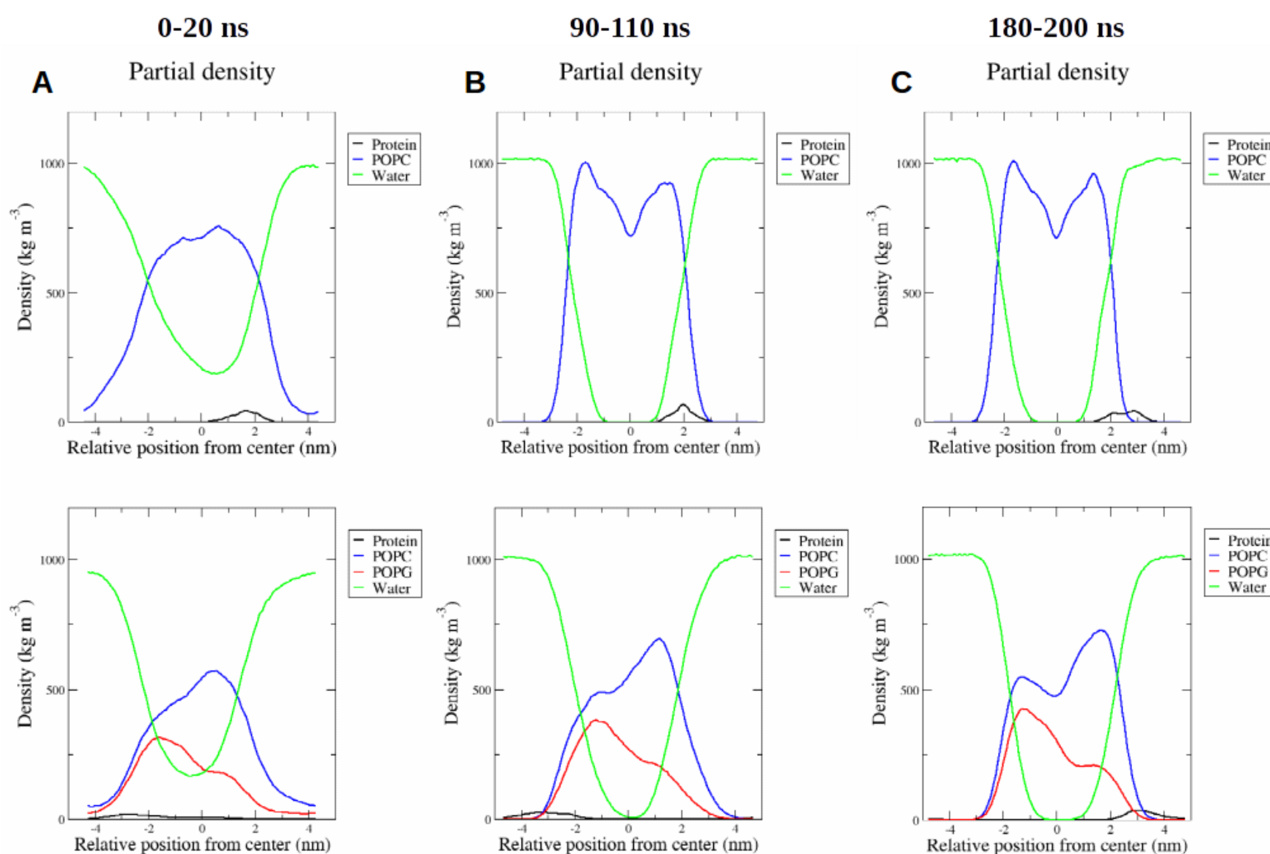


Fig. 4 Mass density profile calculated for the two MD simulations, POPC (top) and POPC:POPG (bottom) during three representative time windows: A) 0-20 ns, B) 90-110 ns and C) 180-200 ns.

Antibacterial activity of the fragment 9-19 of human thymosin β4

TTβ4_{9-19} was tested at concentrations ranging from 25 to 0.07 mg/ml against Gram positive and Gram negative pathogens. The antibacterial activity of the fragment, expressed as minimum inhibitory concentrations (MICs) against planktonic cells of staphylococcal and *P. aeruginosa* reference strains is listed in Table 1. The Tβ4 fragment has been found to interfere with microbial growth of all tested strains. In particular it showed the highest activity against *S. aureus* ATCC 29213 with a MIC value of 6.2 mg/ml. We also reported the data in vitro concerning MIC values of LL37 and bovine lactoferrin as examples respectively of active peptide (positive control) and poorly active peptide (negative control).

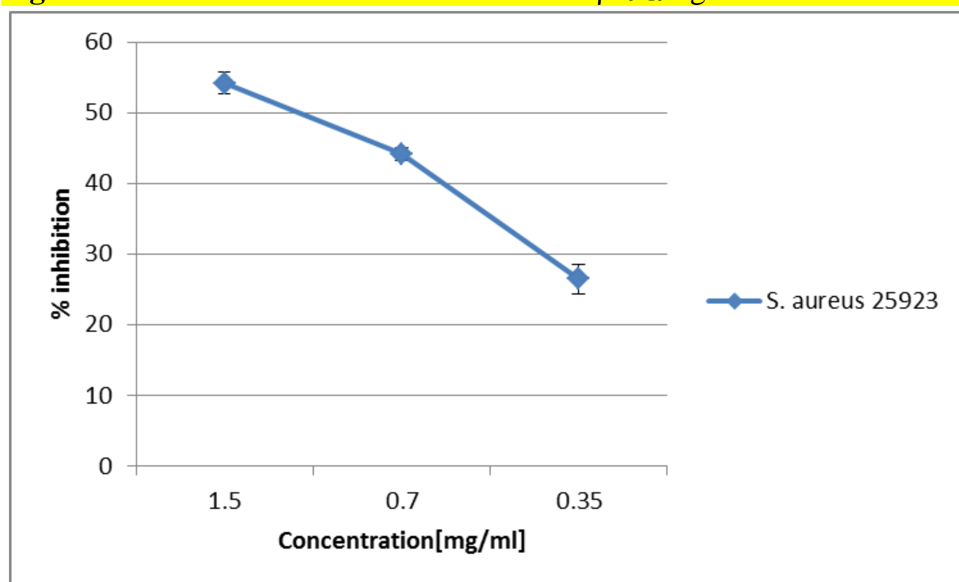
Table 1 Activity of fragment 9-19 of human Tβ4 expressed as MIC, minimum inhibitory concentrations (mg/ml)

MIC (mg/ml)			
Reference strains	**Tβ4 $_{9\text{-}19}$**	**Bovine Lactoferrin**	**LL37**
S. aureus ATCC 29213	6.2	>25	$50x10^{-3}$
S.aureus ATCC 25923	12.5	>25	$50x10^{-3}$
S.aureus ATCC 6538	12.5	>25	$50x10^{-3}$
S. epidermidis RP62A	12.5	>25	$50x10^{-3}$
P.aeruginosa 15442	12.5	>25	$50x10^{-3}$

Interference with biofilm formation

The interference with biofilm formation of human Tβ4$_{9\text{-}19}$ against staphylococcal reference strains as *S. aureus* 25923 and *P. aeruginosa* 15442 was observed. The inhibition against *S. aureus* ATCC 25923 strain was evaluated at the sub-inhibitory concentrations of 1.5, 0.7 and 0.3 mg/ml of the tested peptide. The activity was very relevant at the concentration of 1.5 mg/ml for which the inhibition percentage was 54.2%. At the lowest concentrations the degree of inhibition is reduced following a dose dependence (Fig.5). *P. aeruginosa* strain 15442 was much less susceptible to the inhibitory activity of Tβ4$_{9\text{-}19}$, showing at 3 mg/ml an inhibition percentage of 31.4%.

Fig.5 Interference with biofilm formation of Tβ4_{9-19} against *S. aureus* 25923 (% inhibition)



Discussion

The synthetic fragment 9-19 of human Tβ4 shares some features with many antimicrobial peptides, such as an overall positive charge (+1) and a 27% of hydrophobic residues (Hancock and Lehrer 1998; Wang and Wang 2004; Zasloff 2002). Most of known AMPs, have a cationic amphiphilic alpha-helical structure and they are able to target the cytoplasmic membrane and to provoke cell death by osmotic shock (Di Luca et al. 2014). As previously described, also the peptide SP1 from the sea urchin *P.lividus* has a hydrophobic region of three non polar residues and cationic and polar residues at both ends, which renders the peptide soluble in aqueous solution and provide a binding site for bacterial membranes (Schillaci et al. 2014). These structural features differently from most AMPs, are designed as transmembrane mimetic models (Chan et al. 2004; Liu and Deber 1998; Stark et al. 2002). SP1 MD simulations have shown that Tβ4_{9-19} has a structure constituted by a central hydrophobic region and by the presence of peripheral charged residues. Another example of non-amphipathic antimicrobial peptide is Dermaseptin S9, produced by the skin of the South American hylid frog, *Phyllomedusa sauvagei*; this peptide contains, centrally located, a

hydrophobic core, with a high affinity toward the bacterial membrane (Lequin et al., 2006) and has a microbicidal effect by perturbing the bacterial membrane (Auvynet et al. 2008).

The *in vitro* studies of Tβ$4_{9\text{-}19}$ showed an activity against planktonic forms of *S. aureus* and *P. aeruginosa* at high concentration (12.5 or 6.2 mg/ml). A weak antibacterial activity *in vitro* in the order of mg/ml was also found for a different Echinoderm, *Holothuria tubulosa* (Schillaci et al. 2013) and for some human antimicrobial peptides like lactoferrin (Andrade et al. 2014).

The RMSD plot (Fig 1) shows that Tβ4 9-19 presents peculiar conformations in bulk water solution in a narrow time period (Fig. 2). However a cluster analysis showed that, near the surface of the both membrane models, POPC (mammalian) and POPC:POPG (bacterial), the peptide never assumes a preferred conformation, presumably due to the presence of electrostatic and van der Waals interactions.

However Tβ$4_{9\text{-}19}$ resulted effective in biofilm formation inhibition at lower concentrations than MIC observed for the planktonic forms. The ability to affect biofilm formation of *S. aureus* 25923 at 3.1 mg/ml was very similar to the one observed previously for SP1 but it was less active than SP1 in preventing *P. aeruginosa* biofilm, (inhibition of 31.5% versus 73% respectively) at the concentration of 3.1mg/ml.-

The prevention of biofilm formation, rather than its elimination, remains the more effective way to contrast the growth, as a sessile community of many pathogens. The ability of AMP to prevent biofilm formation might be due to different mechanisms of action: a) an interference with the initial adhesion for modifications of the microbial or biological or artificial surfaces, b) the killing of the early bacterial colonizers, or c) the inhibition of Quorum Sensing (QS), i.e. the intercellular communication system involved in biofilm formation (Batoni et al. 2011). As reported in BaAMPs, the novel database of AMPs specifically assayed against microbial biofilms, a number of natural, semi-synthetic and synthetic AMPs resulted effective against microbial biofilms especially in

preventing their formation, but they are less effective to eradicate mature biofilms (Di Luca et al. 2015). Complete structured biofilms are intrinsically resistant even to AMPs due to a combination of factors, in particular the presence of exopolysaccharides (EPSs) and other extracellular biofilm molecules (amyloids, extracellular DNA) that interfere with the AMPs activity (Di Luca et al. 2014; Rabin et al. 2015).

The overall results showed in this study suggest however, that the fragments 9-19 of human Tβ4, as its analogous from the sea urchin, might be a good platform to design new synthetic or recombinant derivatives with modified chemical-physical properties, with an improved antimicrobial and antibiofilm activity against pathogens (Brogden and Brogden 2011; Huang et al. 2010).

The Authors intend to continue the investigation of AMPs–lipid bilayer (model of erythrocyte and bacterial membrane) or biofilm-interaction through the *in silico* analysis of the binding among the new synthetic or recombinant derivatives and the biofilm matrix molecules.

New antimicrobial agents that interfere with the adhesion (Cascioferro et al. 2014a), the first step of pathogenesis and biofilm formation of *Staphylococcus* spp. and *P. aeruginosa,* could have a great impact, considering that these opportunistic pathogens are responsible of several chronic infections resistant to the most common conventional antibiotics (Percival et al. 2015). A previous study demonstrated that a combined formulation of human Tβ4 and silver sulfadiazine resulted in a more rapid healing of acute wound compared to the single activities of both compounds (Suman et al. 2012). A potential topical application of 9-19 fragments of thymosin or their derivatives in the treatment of infected wounds could be suggested.

The combination of conventional antibiotics and novel anti-biofilm agents is expected to be effective as a new strategy for the treatment of biofilm-associated infections and to tackle the antibiotic resistance.

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