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Hydroxy-tryptophan containing derivatives of tritrpticin: Modification of antimicrobial activity and membrane interactions ☆

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ABSTRACT

Tritrpticin is an antimicrobial peptide with a strong microbicidal activity against Gram-positive and Gram-negative bacteria as well as fungi. The 13-residue peptide is essentially symmetrical and possesses a unique cluster of three Trp residues near the center of its amino acid sequence. The mechanism of action of tritrpticin is believed to involve permeabilization of the cytoplasmic membrane of susceptible bacteria. However it has been suggested that intracellular targets may also play a role in its antimicrobial activity. In this work the mechanism of action of several tritrpticin derivatives was studied through substitution of the three Trp residues with 5-hydroxy-tryptophan (5OHW), a naturally occurring non-ribosomal amino acid. Although it is more polar, 5OHW preserves many of the biophysical and biochemical properties of Trp, allowing the use of fluorescence spectroscopy and NMR techniques to study the interaction of the modified peptides with membrane mimetics. Single or triple 5OHW substitution did not have a large effect on the MIC of the parent peptide against *Escherichia coli* and *Bacillus subtilis*. However, the mechanism of action was altered by simultaneously replacing all three Trp with 5OHW. Our results suggest that the inner membrane of Gram-negative bacteria did not constitute the main target of this particular tritrpticin derivative. Since the addition of a hydroxyl group to the indole motif of the Trp residue was able to modify the mechanism of action of the peptides, our data confirm the importance of the Trp cluster in tritrpticin. This work also shows that 5OHW constitutes a new probe to modulate the antimicrobial activity and mechanism of action of other Trp-rich antimicrobial peptides. This article is part of a Special Issue entitled: NMR Spectroscopy for Atomistic Views of Biomembranes and Cell Surfaces. Guest Editors: Lynette Cegelski and David P. Weliky.

1. Introduction

Antimicrobial peptides (AMPs) have been the object of intense research efforts during the past three decades [1–3]. As an integral part of the innate immune systems of most living organisms (bacteria, plants, insects and animals) AMPs have been identified as a promising class of molecules with considerable therapeutic potential due to their broad spectrum of activity against bacteria, fungi, parasites, viruses and even cancer cells [4,5]. Currently more than 5000 antimicrobial peptide sequences have been identified including naturally occurring sequences, peptide analogs and predicted sequences [6,7]. However, the original promise of a new family of antibiotics based on this type of host-defense molecules has encountered several obstacles. Some of these problems include the toxicity of AMPs towards eukaryotic cells, the loss of activity at physiological pH and salt concentrations, as well as their susceptibility to proteolytic degradation [4]. Initial attempts to modulate the microbicidal activity of the peptides have focused on the modification of the amino acid sequence to enhance their antimicrobial activity and improve their selectivity. Simultaneously the mechanism of action of AMPs has remained a fundamental question, which is important in view of the fact that an understanding of this mechanism should allow for the rational design of better and more specific microbicidal agents [8,9]. Numerous studies have attempted to contribute to this question. Most of the models generated agree on the importance of the bacterial membrane for the activity of the peptides, not only because it acts as the first barrier that the peptide needs to overcome but also because the membrane composition and properties can modulate their activity [10,11]. Most of the AMPs have been described as membrane-

Abbreviations: AMPs, antimicrobial peptides; CD, circular dichroism; COSY, correlation spectroscopy; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol); DSC, differential scanning calorimetry; ePC, egg derived phosphatidylcholine; ePG, egg derived phosphatidylglycerol; LUVs, large unilamellar vesicles; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; MLVs, multilamellar vesicles; NCF, nitrocefin; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser spectroscopy; ONPG, 2-nitrophenyl β-D-galactopyranoside; TOCSY, total correlation spectroscopy

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active agents, which implies that their mechanism of action involves the permeabilization or disruption of the bacterial membrane. However the presence of several AMPs without apparent membrane perturbing effects, yet strong antimicrobial activities, generated proposals for an alternative mechanism of action involving intracellular targets such as DNA, RNA or proteins [12,13].

Tritrpticin is part of the cathelicidin family of AMPs; it has strong antimicrobial activity against various bacteria and fungi [14]. This 13 amino acid peptide is part of the Arg (R)-Trp (W) rich family of AMPs [15], due to its high content of Arg (30.8%) and Trp (23.1%) residues. The sequence of tritrpticin (Table 1) is almost symmetrical and possesses an unusual hydrophobic core formed by three sequential W residues. Tritrp1, the C-terminally amidated version of this peptide, has an increased net positive charge (from +4 to +5), and this simple modification enhanced the antimicrobial activity of the peptide due to the higher electrostatic attraction towards negatively charged bacterial membranes surfaces [16]. Several tritrpticin and Tritrp1 analogs have been developed and studied, showing the importance of residues such as Pro, Arg and Trp in the antimicrobial activity [17–21]. Conservation of the hydrophobic and aromatic character of the peptide's core is an important determinant of the antimicrobial activity of this peptide [16,18]. The mechanism of action of tritrpticin is believed to involve pore formation in bacterial membranes. This notion is based on several biophysical studies of the interaction of tritrpticin with model membranes, as well as on membrane depolarization studies with *Staphylococcus aureus* and *Escherichia coli* [18–20,22–25]. However intracellular targets are also considered as part of tritrpticin's mechanism of action [26].

Numerous studies of AMPs are focused on the engineering of new peptide analogs that resemble the naturally occurring peptides, by modification of the original peptide sequences with natural amino acids, D-amino acids, β-amino acids and other unnatural or non-coded amino acids, see for example [27–32]. In this work Tritrp1 was modified through substitution of its Trp residues with 5-hydroxytryptophan (5OHW). This naturally occurring amino acid is well known as a metabolic intermediate in the biosynthesis of serotonin and melatonin [33]. While 5OHW is not used in the ribosomal biosynthesis of proteins, it has been deployed in several studies as a fluorescence enhancement probe, due to its distinct fluorescence spectroscopic properties. 5OHW exhibits a red shifted absorbance in comparison to Trp, by the presence of a shoulder at 310 nm. This unique spectroscopic characteristic has allowed studies of several protein–protein interactions, protein oligomerization states and conformational changes, as well as investigations of the folding and stability of proteins [34–36].

In the present study we determined whether 5OHW substitutions influenced the antimicrobial activity of Tritrp1; the sequence of the peptides studied are shown in Table 1. Secondly, we studied the mechanism of action of these Tritrp1 derivatives by fluorescence, circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy, differential scanning calorimetry (DSC) and various other assays. Our results revealed that the substitutions of individual or all three Trp residues by 5OHW did not alter the antimicrobial activity of the peptide. However the mechanism of action was significantly changed when all three Trp residues were substituted simultaneously.

Table 1
Sequence of Tritrp1 and its derived peptides containing 5-hydroxytryptophan (5OHW).

Peptide	Sequence
Tritrp1	VRRFPWWWPFLRR-NH_2
HW	VRRFP^{5OH}W^{5OH}W^{5OH}WPFLRR-NH_2
HW1	VRRFP^{5OH}WWWPFLRR-NH_2
HW2	VRRFPW^{5OH}WWPFLRR-NH_2
HW3	VRRFPWW^{5OH}WPFLRR-NH_2

2. Materials and methods

2.1. Materials, bacterial strains and peptides

Nitrocefin (NCF) and 2-Nitrophenyl β-D-galactopyranoside (ONPG) were obtained from EMD Millipore Corporation (Billerica, MA) and Sigma-Aldrich (St. Louis, MO) respectively. [Methyl-^{3}H]-thymidine and [5-^{3}H]-uridine were purchased from Perkin Elmer (Waltham, MA), and [ring-2-5-^{3}H]-L-histidine was obtained from Moravek Biochemicals and Radiochemicals (Brea, CA). Sodium dodecyl sulfate (SDS) was purchased from EMD Chemicals Inc. (Gibbstown, NJ) and SDS-d_{25} was obtained from Cambridge Isotopes Laboratories (Andover, MA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

All Tritrp1-derived peptides were purchased from AnaSpec Inc. (Fremont, CA). The peptides were synthesized by solid phase methods and their purity (>95%) and molecular weight were checked by HPLC and mass spectrometry, respectively. Melittin, purified from honey bee venom, was purchased from Sigma-Aldrich (St. Louis, MO).

E. coli ATCC 25922 was purchased from the American Type Culture Collection (Manassas, VA). *E. coli* CGSC 4908 was obtained from the *E. coli* genetic stock center (CGSC) at Yale University (New Haven, CT). *E. coli* ML35p and *Bacillus subtilis* 168 were kindly provided by Dr. Robert Lehrer at UCLA David Geffen School of Medicine and Dr. Sui-Lam Wong at University of Calgary, respectively.

2.2. Antibacterial activity

The antimicrobial activity of Tritrp1-derived peptides was measured as the minimal inhibitory concentration (MIC) according to the standard broth microdilution method previously described by Wiegand et al. [37]. Bacteria (*E. coli* ATCC 25922 and *B. subtilis* 168) were grown in Mueller–Hinton broth (MHB), which was incubated at 5×10^5 cfu/ml in the presence of two-fold dilutions of MHB-dissolved peptides (0.25 to 128 μM) in a 96-well polypropylene plate, and incubated overnight at 37 °C. The MIC values reported correspond to the minimal peptide concentration where bacterial growth was not observed. In order to establish the minimal bactericidal concentration (MBC) 10 μl from the first three wells without bacteria growth for each peptide in the MIC plate were diluted 1:10^6 in MHB and plated (100 μl) in MHB-agar plates. After incubation overnight at 37 °C the presence of colonies was established. The MBC values reported correspond to the minimal peptide concentration where no colony formation was detected.

2.3. Multilamellar vesicles (MLVs) and large unilamellar vesicles (LUVs) preparation

MLVs were prepared by adding the necessary volume of DPPG or DPPC-chloroform stock solutions (Avanti Polar Lipids, Alabaster, AL) into a glass vial in order to have 0.5 mg of lipids. The organic solvent was initially removed by evaporation in a stream of nitrogen gas and the remaining solvent was evaporated under vacuum overnight. The dry lipids were resuspended with warm Tris-buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4) by vigorous vortexing. For DSC studies, Tritrp1-derived peptides in aqueous solution were added to the MLVs suspension to achieve a peptide:lipid molar ratio of 1:10.

LUVs were made by initially preparing MLVs, as described previously, with the necessary volume of ePC, ePG or cholesterol–chloroform stock solutions (Avanti Polar Lipids, Alabaster, AL) to obtain the desired ratio. Two types of LUVs were prepared, ePC:ePG (1:1 molar ratio) and ePC:cholesterol (2.5:1 molar ratio). The MLVs were freeze–thawed five times using liquid nitrogen, followed by extrusion through two 0.1 μm polycarbonate filters (Nucleopore Filtration Products, Pleasonton, CA) using a mini-extruder apparatus (Avanti Polar Lipids, Alabaster, AL) to generate LUVs with a 100 nm diameter. Calcein containing LUVs were prepared by resuspending the dry lipids in Tris-buffer supplemented

with 70 mM calcein. After LUV preparation, free calcein was removed by gel filtration using a Sephadex G-50 column. The concentration of lipids in the LUVs was measured by the Ames phosphate assay [38], performed in triplicate.

2.4. Tryptophan fluorescence, blue shift ($\Delta\lambda_{emi}$) and acrylamide quenching

The tryptophan fluorescence was monitored using a Varian Cary Eclipse Fluorimeter (Agilent Technologies, Santa Clara, CA) equipped with a multicell sample holder and temperature control set to 25 °C. The peptide (1 μM) in Tris-buffer was excited at 280 nm (slit width 10 nm) and the emission spectra were measured at 300–500 nm (slit width 10 nm) in the presence or absence of LUVs (100 μM). The difference in the maximum emission wavelength between the absence and presence of LUVs is referred to here as the blue shift ($\Delta\lambda_{emi}$).

For the acrylamide quenching experiments sequential additions of 5 μl of a 4 M acrylamide stock solution (up to 0.15 M final concentration) were made to the sample containing the peptide (1 μM) and LUVs (100 μM). The tryptophan fluorescence was recorded after each addition of acrylamide and fluorescence intensity changes were analyzed through Stern–Volmer plots from where the quenching constants (*Ksv*) were calculated from the Eq. (1):

$$Fo/F = 1 + Ksv[Q] \quad (1)$$

where *Fo* is the initial fluorescence of the peptide and *F* is the fluorescence following the addition of the quencher *Q*.

2.5. Circular dichroism (CD) spectroscopy

The secondary structure of the Tritrp1-derived peptides (50 μM) in buffer (10 mM Tris pH 7.4) and in the presence of SDS micelles (35 mM) was assessed using a Jasco J-810 spectropolarimeter (Jasco Inc. Easton, MD). The far-UV spectra were recorded between 190–260 nm in a 1 mm pathlength cuvette at room temperature, under a constant flow of nitrogen. The ellipticity was registered every 0.1 nm, with a scan rate of 200 nm/min and 0.1 nm bandwidth. The final spectra represent the average of 10 consecutive scans.

2.6. ^{1}H NMR studies and structure determination

For NMR studies peptides (~3 mg) were dissolved in 9:1 H_2O:D_2O, and for the peptides interacting with micelles, d_{25}-SDS was added at 1:100 (peptide:SDS ratio) ensuring a ratio of one peptide molecule per micelle. A one-dimensional (1D) ^{1}H NMR spectrum was acquired using 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as internal chemical reference and water suppression was achieved by excitation sculpting [39]. Two dimensional nuclear Overhauser enhancement and exchange spectroscopy (NOESY), correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) spectra were obtained for the peptides with SDS micelles with a mixing time of 0.25 and 0.094 s for the NOESY and TOCSY experiments respectively. All NMR experiments were acquired on a 600 MHz Bruker Avance spectrometer (Bruker Corporation, Milton, ON) and were processed with the NMRPipe software package [40].

The spectral analysis was performed using NMRView 5.2.2.01 [41], and chemical shift assignments were done according to Wüthrich [42]. Structural calculations were performed using ARIA 1.2 [43]. Additionally a manually modified version of the topology files in order to include the 5-hydroxy-tryptophan was used. Broad dihedral restraints were imposed on all of the non-Gly residues to keep the initial *phi* and *psi* angles within allowable regions of the Ramachandran plot. In the final ARIA calculation run 100 structures were calculated and the 20 lowest energy structures were used for analysis using Procheck [44] and visualized in MOLMOL [45].

2.7. Differential scanning calorimetry (DSC)

The DSC experiments of MLVs and the effects of the Tritrp1-derived peptides on the thermotropic phase transition were studied using a Microcal high sensitivity VP-DSC instrument (Malvern Instruments Inc. Westborough, MA). The final concentration of MLVs for each DSC experiment was 0.5 mg/ml. Five heat scans (10 °C/h), between 20–60 °C were performed for the experiments involving DPPC and DPPG. The analysis of the fifth heating scan was performed with the Microcal Origin Software (version 7.0).

2.8. Calcein leakage

Release of calcein from LUVs was registered by fluorescence spectroscopy, which was measured in a Varian Cary Eclipse Fluorimeter (Agilent Technologies, Santa Clara, CA). Calcein containing LUVs (10 μM) were incubated in Tris-buffer and the fluorescence was registered at 520 nm (slit width 10 nm) during 1 min, with excitation at 490 nm (slit width 10 nm). Next the peptide was added at different concentrations and the fluorescence was registered for another 15 min. At this point all LUVs were solubilized by 0.02% Triton X-100 and the fluorescence registered for another minute. The leakage percentage was calculated according to Eq. (2):

$$\%\ \text{Calcein leakage} = 100 \times (I - I_o)/(I_t - I_o) \quad (2)$$

where I_o is the fluorescence before the addition of the peptide, I is the fluorescence at 10 min after the addition of the peptide, and I_t is the fluorescence after the addition of Triton X-100.

2.9. E. coli outer and inner membrane permeabilization

The peptide-induced permeabilization of the outer and inner membrane in Gram-negative bacteria was recorded as described by Epand et al. [46]. The method uses the *E. coli* strain ML35p, which in addition to being constitutive for cytoplasmic β-galactosidase, also expresses the plasmid-encoded periplasmic β-lactamase enzyme, and lacks the *lac* permease. The membrane impermeable ONPG substrate is hydrolyzed by the cytoplasmic β-galactosidase and can be monitored by absorbance at 420 nm. Similarly, the impermeable NCF is the substrate of the periplasmic β-lactamase, for which the hydrolysis can be followed by absorbance at 490 nm.

E. coli ML35p was grown at 37 °C in Luria broth (LB) media from a single colony until OD_{600} ~ 0.6. The cells were collected and washed three times with incubation buffer (10 mM Na^+-phosphate pH 7.4, 100 mM NaCl and 300 μg/ml LB) and added to a final OD_{600} of 0.3 in the presence of ONPG (0.5 mM) or NCF (30 μM) in a 96-well plate, together with peptide concentrations of 0.2 to 20 μM. The absorbance of the wells was measured at 420 and 490 nm for ONPG and NCF experiments, respectively, every 2 min during 60 min, in a Perkin Elmer Victor™ X4 Multilabel Plate Reader (Waltham, MA) with shaking and temperature control at 37 °C.

2.10. DNA mobility/gel retardation assay

In order to evaluate peptide binding to DNA the agarose gel retardation assay was used [47]. The plasmid DNA pET19b was purified from *E. coli* DH5α cells using the Qiagen Plasmid purification kit (Qiagen Inc. Valencia, CA). The DNA concentration and purity were measured at A_{260} and A_{260}/A_{280} respectively. Plasmid DNA (100 ng) was incubated at room temperature with increasing amounts of peptides, corresponding to peptide/DNA ratios from 0–10 (w/w), in binding buffer (5% glycerol, 10 mM Tris, 1 mM EDTA, 1 mM DTT, 20 mM KCl, 50 μg/ml BSA, pH 8.0). After incubation for 1 h, loading dye 5× (10% Ficoll 400, 10 mM Tris, 50 mM EDTA, 0.25% bromophenol blue, pH 7.5) was added to the mixture and loaded onto a 1% agarose gel in Tris-acetate-

EDTA (TAE) buffer containing 50 μl ethidium bromide for visualization. The gels were run at 100 V for 30 min and its image taken in a Bio-Rad Geldoc XR (Bio-Rad Laboratories, Hercules CA).

2.11. Macromolecular synthesis inhibition

The inhibition of DNA, RNA and protein synthesis in *E. coli* was evaluated using a modified protocol previously described by Patrzykat et al. [48]. Briefly, *E. coli* CGSC 4908 (auxotroph for thymidine, uridine and L-histidine) was grown overnight at 37 °C from a single colony. The overnight culture was diluted 1:10^3 and grown at 37 °C to the exponential phase (OD_{600} ~ 0.3). At this point the cells were collected and resuspended in M9 minimal media [49], to OD_{600} 0.3. Aliquots (300 μl) of the cell suspensions were supplemented with 9 μl of either [^{3}H]-thymidine, [^{3}H]-uridine or [^{3}H]-L-histidine and 100 mg/l of the other two unlabeled precursors. After 5 minutes incubation at 37 °C peptides were added at specific concentrations (0, 0.8, 2, 4 and 8 μM), followed by incubation for 30 more minutes at 37 °C. At time points of 0, 5, 15 and 30 min 50 μl samples were taken from the cultures and incubated in 5 ml 10% trichloroacetic acid (TCA) supplemented with 200 mg/l of all three unlabeled precursors for 40 min on ice, followed by 15 min incubation at 37 °C. The DNA, RNA and proteins were collected over vacuum using 25 mm nucleic acid and proteins transfer filters from Whatman GmbH (Dassel, Germany). The filters were rinsed 10 times with cold 10% TCA and dried at 70 °C. Each filter was placed in a scintillation vial with 10 ml of BCS-NA non-aqueous biodegradable counting scintillant (GE Healthcare, Piscataway, NJ) and the radioactive counts recorded for 1 min in a Beckman LS6500 multi-purpose scintillation counter (Beckman Coulter, Brea, CA).

In order to establish how these peptide concentrations affect the growth of *E. coli* CGSC 4908, a separate growth assay was performed. The bacterial cells were prepared as described before in M9 minimal media supplemented with 200 mg/l of all three unlabeled precursors and incubated at 37 °C. At each time point, samples of 5 μl were diluted 1:4 × 10^4 in water and 100 μl plated in LB-agar plates. After incubation overnight at 37 °C, the number of colonies was counted.

3. Results

3.1. Antibacterial activity

The MIC and MBC activities of the Tritrp1-derived peptides are presented in Table 2. All peptides exhibited a higher antimicrobial activity towards the Gram-positive organism *B. subtilis* 168 in comparison to the Gram-negative *E. coli* ATCC 25922. In addition, the antibacterial activity of all peptides after 18 h was shown to be bactericidal rather than bacteriostatic, as indicated by the similar MIC and MBC values. The peptide Tritrp1 had a MIC of 4 μM against *E. coli*, which corresponds well to previously reported data [16,20]. This MIC value was comparable to the 2–4 μM obtained for melittin, a known strong membrane-lytic peptide [50]. Individual or triple substitutions of Trp residues by 5OHW did not have any large effects on the antimicrobial activity of the Tritrp1 analogs.

Table 2
Antimicrobial activities of Tritrp1-derived peptides and melittin reported as minimal inhibitory and bactericidal concentrations (μM), MIC and MBC respectively.

	E. coli ATCC 25922		*B. subtilis* 168	
	MIC	MBC	MIC	MBC
Tritrp1	4	4	1	1
HW	4–8	4–8	0.5–1	0.5–1
HW1	4–8	4–8	2	2
HW2	8	8	1	1
HW3	4–8	4–8	1	1
Melittin	2–4	2–4	0.25–0.5	0.25–0.5

The values presented are the results of three independent experiments.

3.2. Tryptophan fluorescence spectroscopy and blue shift measurements

The fluorescence excitation and emission spectra for Tritrp1 and its derived peptides in buffer are depicted in supplementary Fig. S1, while the maxima wavelengths obtained from these spectra are presented in Table 3. Tritrp1 exhibited a fluorescence emission maximum at 349 nm, while the excitation maximum was located at 280 nm. These values are characteristic for Trp in a polar environment (supplementary Fig. S2). In comparison, the peptide with three 5OHW residues (HW) showed an excitation maximum wavelength at 311 nm, which correspond to the 310 nm shoulder observed for 5OHW amino acid in buffer (supplementary Fig. S2). However, the emission maximum of HW located at 398 nm represents an extremely red shifted emission in comparison to the maximum for the 5OHW in buffer located at 340 nm (supplementary Fig. S2). For the individually substituted peptides (HW1, HW2 and HW3) in buffer the excitation and emission maxima wavelength were similar, located around 280 and 338 nm respectively.

The fluorescence emission spectrum of the Trp side chain is highly sensitive to the polarity of the environment, and its maximum wavelength ($\lambda_{emi,\ max}$) can be used to study the interactions between Trp-containing peptides and membranes. During peptide–membrane interactions, the exposure to a more hydrophobic environment induces a decrease in the $\lambda_{emi,\ max}$ of the Trp side chain, which is known as blue shift. In comparison to Trp, the 5OHW side chain is less sensitive to solvent polarity changes, thus limiting the usefulness of 5OHW as a probe for studying peptide–membrane interactions [51]. The fluorescence maxima wavelength and the blue shift for all Tritrp1-derived peptides upon interaction with membranes are shown in Table 3 and supplementary Fig. S1. Tritrp1 exhibited a large blue shift (12 nm) in the presence of ePC:ePG vesicles, while a smaller blue shift (5 nm) was observed with ePC:Chol vesicles. In contrast, the HW peptide exhibited a similar, although small, blue shift for both ePC:ePG and ePC:Chol vesicles. In the case of single substituted peptides, no differences were observed between the presence of ePC:ePG and ePC:Chol vesicles. Both vesicles induced only a minor blue shift (0–2 nm).

3.3. Acrylamide quenching

Complementary to the fluorescence blue shift experiments, solvent accessibility measurements of the Trp side chain can provide information about the peptides environment when in buffer or in the presence of membranes. Acrylamide quenches the fluorescence of solvent exposed Trp residues. Its uncharged nature allows quenching studies involving negatively charged membranes, which can influence the behavior of ionic quenchers such as Cs^+ and I^- [52]. Both amino acids Trp and 5OHW are similarly quenched by acrylamide as shown in the supplementary Fig. S3. The acrylamide quenching levels (represented by *Ksv*) for the Tritrp1-derived peptides in buffer or in the presence of lipid vesicles are presented in Fig. 1. The ePC:ePG vesicles exhibit a net negatively charged surface resembling bacterial cell membranes, while the use of a zwitterionic phospholipid (PC) and cholesterol simulated the electrically neutral and fluidity/rigidity characteristics of a eukaryotic membrane. All

Table 3
Maximum fluorescence excitation ($\lambda_{exc,\ max}$) and emission ($\lambda_{emi,\ max}$) wavelengths (nm) for the Tritrp1-derived peptides in Tris-buffer and blue shift ($\Delta\lambda_{emi}$ [a]) upon binding to LUVs. Blue shift results are average ± S.D. (n = 3).

	Tris-buffer		Blue shift	
	$\lambda_{exc,\ max}$	$\lambda_{emi,\ max}$	ePC:ePG	ePC:Chol
Tritrp1	280	349	12 ± 1	5 ± 2
HW	311	398	4 ± 2	1 ± 4
HW1	280	337	0 ± 1	1 ± 0
HW2	278	338	1 ± 1	1 ± 0
HW3	280	339	2 ± 1	1 ± 0

[a] $\Delta\lambda_{emi} = \lambda_{emi,\ max}$ (buffer) − $\lambda_{emi,\ max}$ (LUVs).

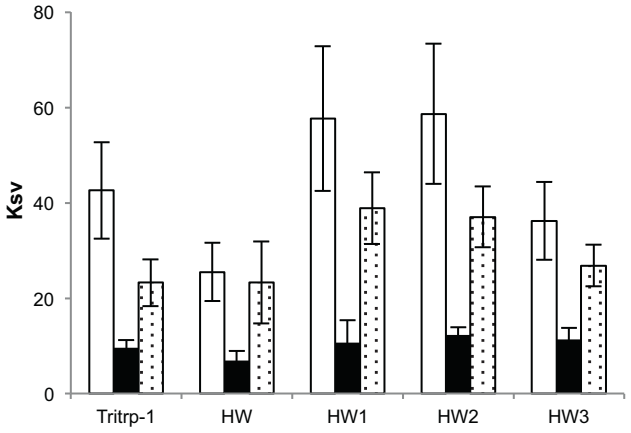

Fig. 1. Stern–Volmer constants (*Ksv*) for the Tritrp1-derived peptides as determined from the acrylamide quenching experiments in different media: buffer (*white*), in the presence of ePC:ePG LUVs (*black*) and ePC:cholesterol LUVs (*dotted*). Results are average ± S.D. (n = 3).

peptides interacted with the negatively charged ePC:ePG vesicles, exhibiting small *Ksv* values in comparison to those observed in buffer. In contrast, higher *Ksv* values were observed in the presence of ePC:Chol vesicles. Interestingly the *Ksv* of HW in buffer was considerably lower than all other peptides, indicating that this peptide has unique properties.

3.4. Circular dichroism spectroscopy

The far-UV CD spectra of the Tritrp1-derived peptides in buffer and in SDS micelles are presented in Fig. 2. These micelles were selected in order to simulate the negative surface charge of bacterial membranes. According to the appearance of the CD spectra, the peptides could be divided in two groups. The first group was formed by Tritrp1, HW1 and HW3. Their CD profile was dominated by a strong negative band around 226 nm. Upon interaction with SDS micelles the intensity of this negative band was slightly modified, while two positive bands at 207 and 195 nm appeared for Tritrp1 and HW1. The second group was formed by HW and HW2. In buffer HW exhibited two negative bands, a strong band around 205 nm and a weaker band at 230 nm. The presence of SDS micelles only modified the CD signal at 190 nm. HW2 showed weak signals in buffer with negative peaks at 200 and 228 nm. However a considerable conformational change was observed in the presence of SDS micelles due to the presence of negative bands at 190 and 215 nm. These data confirm that all peptides bind to these membrane mimetics.

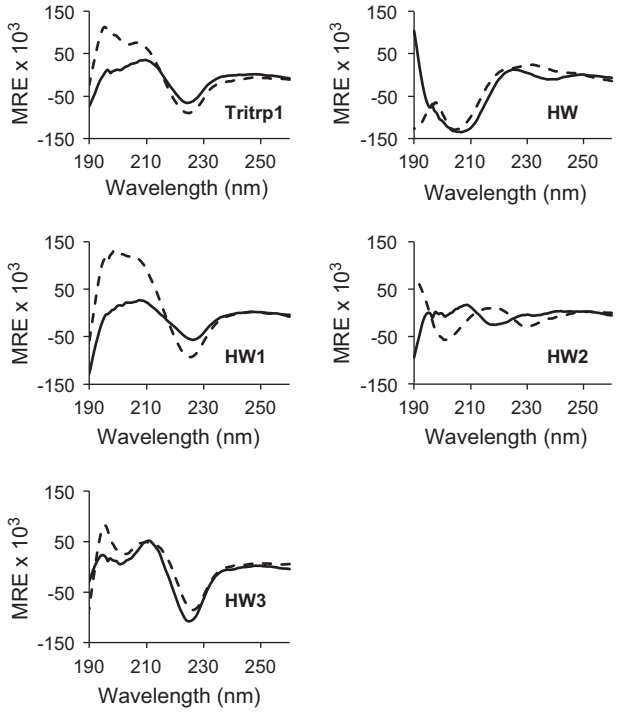

Fig. 2. Circular dichroism far-UV spectra of Tritrp1-derived peptides in aqueous environment (*solid*) and SDS micelles (*dashed*). The experiments were performed in duplicate, representative results are shown.

3.5. ^{1}H NMR studies and structure determination

In addition to the secondary structural information obtained by circular dichroism, 1D and 2D ^{1}H NMR spectroscopy were used to study in detail the 3D-structures induced by the binding of the Tritrp1-derived peptides to the SDS micelles (Fig. 3). ^{1}H NMR studies of Tritrp1 in DPC and SDS micelles have already been reported [17], hence only the 5OHW containing peptides were analyzed. In buffer the peptides HW1, HW2 and HW3 exhibited at least nine resonances in the spectral region corresponding to the Hε1 of the Trp residues (9.0–11.0 ppm). The presence of more than three peaks, corresponding to the indole-NH group of the three Trp residues in tritrpticins, is an indication of multiple conformations co-existing in aqueous solution as described previously [16]. Upon binding to micelles three main signals were clearly dominating the NMR spectra for the single substituted peptides. This indicates that the interaction among the peptides and the micelles favors the formation of a single more stable conformation. For HW, only three resonances were observed in buffer and in the presence of SDS micelles,

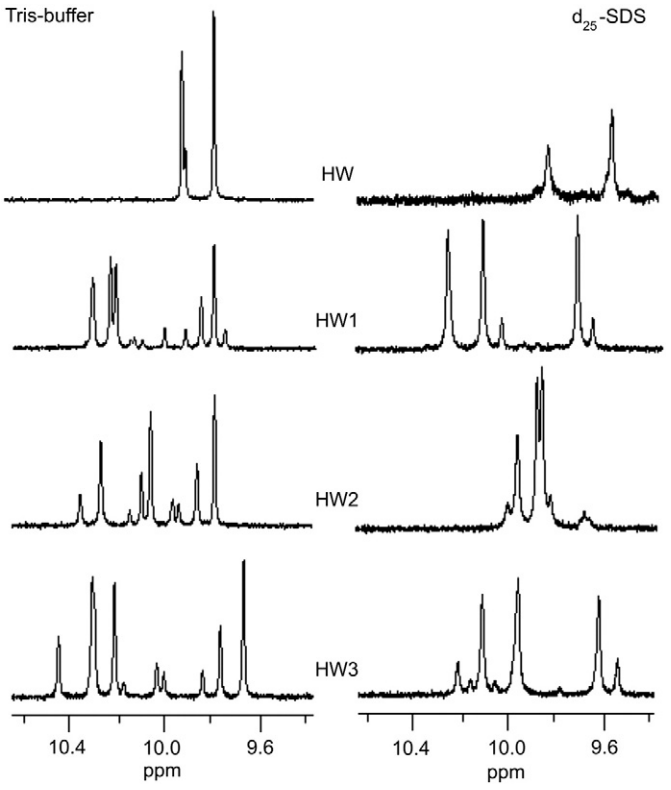

Fig. 3. One-dimensional proton NMR spectra for the Hε1-tryptophan region of Tritrp1-derived peptides in Tris-buffer (*left*) and in the presence of d_{25}-SDS micelles (*right*) at 37 °C.

indicating that only one conformation of the peptide was present in both cases. However, the peak intensities in micelles were reduced, as their linewidths increased, compared to the spectra for the other peptides.

The 3D-structure for the main micelle-bound conformation of the peptides was established by 2D ^{1}H NMR experiments. The structures determined for the single substituted peptides HW1, HW2 and HW3 are depicted in Fig. 4. The structural statistics are provided in the supplementary data (supplementary Table S1). The structure of HW1 in the presence of SDS micelles resembled the elongated boat-like structure reported previously for tritrpticin in DPC micelles [16]. A clear amphipathic configuration emerged from the spatial separation of the polar Arg residues located in the N- and C-terminal regions. These regions faced one side of the molecule, while the hydrophobic core formed by the Trp residues was facing the other side (Fig. 4). In comparison to HW1, HW2 and HW3 exhibited a more compact structure. Both N- and C-terminal regions are located closer to each other, and in the case of HW3 this behavior was even more pronounced. This conformation allows for a more extreme amphipathic structure as depicted in the electrostatic surface charge distribution (Fig. 4).

3.6. Differential scanning calorimetry

DSC of membrane models using DPPG and DPPC phospholipids was performed to study how the peptide–membrane interactions affect the lipid packing in MLVs (Fig. 5). DPPC and DPPG are normally used to emulate different biological membranes. DPPG has a negatively charged head group, which simulates the negative charge observed on the bacterial membrane surfaces. Similarly DPPC is a zwitterionic phospholipid that can be used to simulate the neutral charge of the outer leaflet of eukaryotic membranes [53].

The DPPC lipids exhibited a main phase transition peak at 41.2 °C and a pre-transition peak located at 34.2 °C. Upon binding to DPPC lipids, all peptides modified the pre-transition peak, but the changes in the main transition temperature (T_m) were restricted to +/− 0.1 °C (Fig. 5A). In contrast there was a stronger influence on the lipid packing in DPPG membranes upon interaction with the Tritrp1 and the 5OHW containing peptides (Fig. 5B). DPPG lipids exhibited a main phase transition peak at 40.0 °C, with a pre-transition peak located at 29.8 °C. The pre-transition peak in all cases also disappeared (Fig. 5B), and the T_m of the main transition was maintained between 39.7 °C and 41.2 °C. However, there was a considerable broadening of the main transition peak accompanied by the appearance of shoulders or smaller peaks at lower temperatures. The latter probably represent the formation of peptide–lipid clusters.

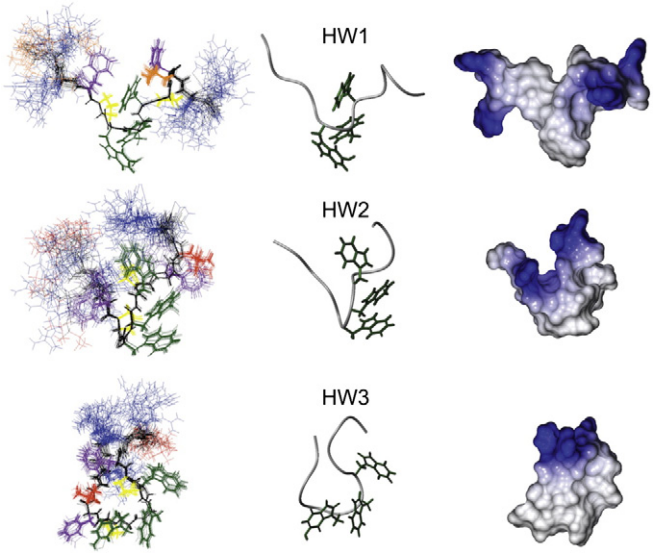

Fig. 4. Solution structures of single substituted Tritrp1-derived peptides (HW1, HW2 and HW3) in d_{25}-SDS micelles. Overlays of the 20 lowest energy structures (*left*), ribbon representation of the backbone and tryptophan residues (*middle*) and electrostatic potential surfaces (*right*) were depicted using the software package MolMol. The structures are overlaid for the backbone atoms 4–11, with RMSDs of 0.096, 0.256 and 0.200 Å, for HW1, HW2 and HW3 respectively.

3.7. Calcein leakage

The ability of the peptides to induce membrane permeabilization was assessed by the calcein leakage method in both ePC:ePG and ePC:Chol LUVs (Fig. 6). Tritrp1 was able to induce substantial leakage of entrapped calcein from ePC:ePG (Fig. 6A) and ePC:Chol (Fig. 6B) vesicles, as previously described [16,20]. In contrast to Tritrp1, when calcein containing ePC:ePG and ePC:Chol vesicles were incubated with the HW peptide almost no calcein leakage was observed (Fig. 6). The individual modifications of the three Trp residues indicated that the location of the substituted residues determine the effect on the permeabilization of the ePC:ePG membrane (Fig. 6A). HW2 and HW3 exhibited a similar, or even slightly higher tendency to induce calcein leakage than Tritrp1, while HW1 induces calcein leakage at an intermediate level between Tritrp1 and HW. In contrast all three individually substituted peptides (HW1, HW2 and HW3) exhibited a similar intermediate level of calcein leakage in ePC:Chol vesicles (Fig. 6B).

3.8. E. coli outer and inner membrane permeabilization

Membrane mimetic systems such as LUVs only allow the simulation of particular characteristics of biological membranes, as they obviously cannot fully represent the highly complex and heterogeneous bacterial membrane. In order to visualize the effect of our Tritrp1-peptides in a more biologically relevant system, the *E. coli* ML35p bacterial strain was used, and permeabilization of its inner and outer membrane was studied by following ONPG and NCF hydrolysis [46].

The inner membrane permeabilization induced by all peptides is shown in Fig. 7 (*left*). Tritrp1 (4 μM) clearly induced a considerable increase in the membrane permeabilization, as detected by a fast rate of ONPG hydrolysis by β-galactosidase, with a lag period of 10 min. This peptide concentration corresponds to the MIC value against *E. coli* ML35p (data not shown). In contrast, triple substitution of the Trp residues by 5OHW completely abolished the inner membrane permeabilization even at a concentration of 20 μM. Individually substituted peptides (HW1, HW2 and HW3) exhibited a similar behavior among them. All three peptides induced membrane permeabilization at concentrations of 4 and 8 μM with a time lag period of 20–40 min.

The outer membrane permeabilization induced by Tritrp1 and its derived peptides is shown in Fig. 7 (*right*). All peptides seemed to induce similar levels of outer membrane permeabilization allowing NCF to reach the periplasmic β-lactamase and hydrolysis to take place (Fig. 7, *right*). However it is important to note that even in the absence of peptides there was a basal level of NCF leakage and hydrolysis, corresponding to a steady increase in the absorbance at 490 nm. Nevertheless it was still possible to observe differences in the permeabilizing ability of the peptides tested. Tritrp1 and HW were the strongest permeabilizing agents for the outer membrane. A concentration of 0.8 μM was sufficient to induce a rapid increase in the absorbance over the basal level. The peptides HW1, HW2 and HW3 exhibited again a similar behavior when compared to each other, inducing permeabilization only at 2 μM or higher concentrations.

3.9. Macromolecular synthesis inhibition

The inhibition of vital intracellular processes such as DNA-, RNA- and protein syntheses due to the action of Tritrp1 and the triple substituted HW peptide are presented in Fig. 8. Only Tritrp1 and HW were studied

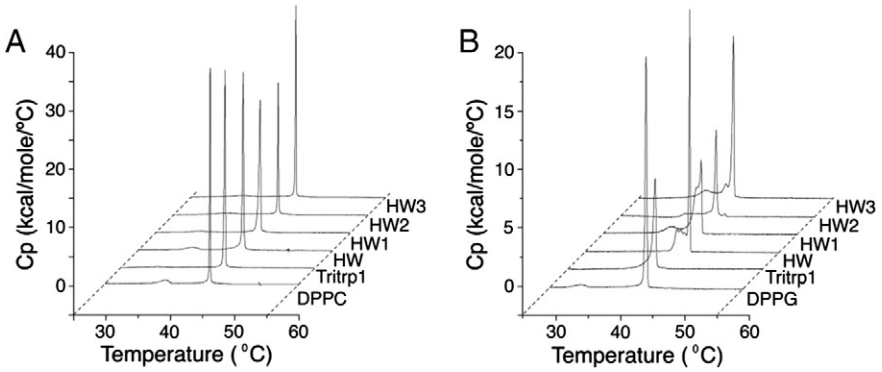

Fig. 5. Differential scanning calorimetry scans of DPPC (A) and DPPG (B) MLVs interacting with Tritrp1-derived peptides at 1:10 (peptide:lipid) molar ratio. Enhancements of the main phase transition for DPPG in the absence or presence of peptides are also depicted in supplementary Fig. S4.

here because of the marked differences observed in their membrane interaction and permeabilization. In contrast, the behavior of HW1, HW2 and HW3 was similar to Tritrp1, as detected in the earlier experiments.

Tritrp1 was able to induce complete inhibition of the synthesis of DNA, RNA and proteins, at peptide concentrations as low as 0.8 μM, corresponding to 1/5 of the MIC value for *E. coli* ATCC 25922. This inhibitory effect was efficient, considering that in the first 5 min of incubation complete inhibition was observed (Fig. 8A, C and E). Higher concentrations of the HW peptide (2 μM) were required in order to achieve complete inhibition of the DNA, RNA and protein synthesis comparable to Tritrp1 (Fig. 8B, D and F). It is important to note that the peptide concentrations used in this experiment did not induce complete cell death over the time frame of these experiments (data not shown).

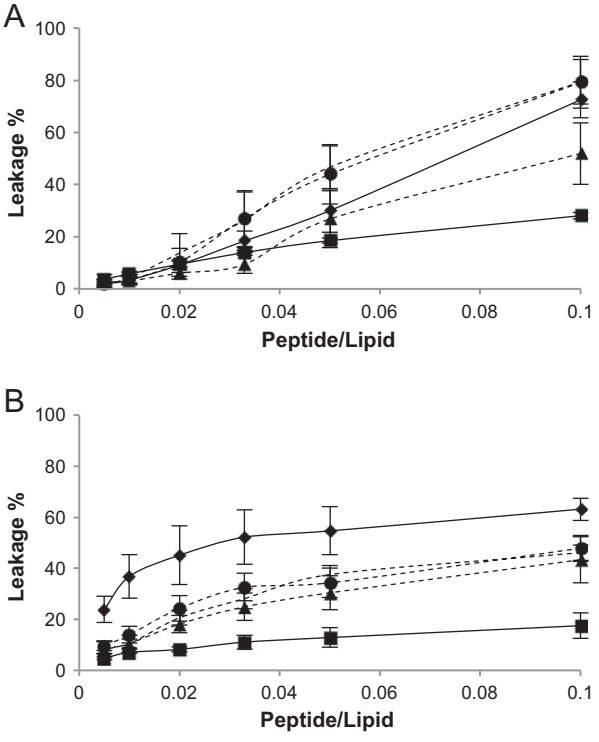

Fig. 6. Calcein leakage percentage for the Tritrp1-derived peptides in the presence of ePC:ePG (1:1) LUVs (A) and ePC:Cholesterol (2.5:1) LUVs (B). The peptides are as follows: Tritrp1 (*solid*, ♦), HW (*solid*, ■), HW1 (*dashed*, ▲), HW2 (*dashed*, ●) and HW3 (*dashed*, −). Results are average ± S.D. (n = 3).

3.10. DNA mobility/retardation assay

Considering that the bacterial membrane might not be the only target of Tritrp1, intracellular DNA has been proposed as a possible antimicrobial targets for some cationic antimicrobial peptides [12,13]. It is expected that due to the cationic nature of Tritrp1 and its derivatives (+5), they are attracted to highly negatively charged molecules such as DNA. The peptide–DNA interactions were studied by following the changes in DNA mobility during agarose electrophoresis. Binding of a peptide to DNA will considerably increase the size of the molecule, affecting or even preventing its mobility through the agarose gel (Fig. 9). We observed, that Tritrp1 was the strongest DNA binder among all peptides; addition of only 200 ng was enough to completely inhibit DNA mobility. The substitution of all Trp for 5OHW residues considerably affected the interaction of the peptides with DNA as shown by the higher HW concentrations required to inhibit DNA migration in the agarose gel. For the individually substituted peptides the location of the Trp substitution influenced the DNA binding ability of the peptide. HW1 was highly affected, while HW2 and HW3 bound somewhat stronger to the plasmid DNA.

4. Discussion

Numerous studies of AMPs have focused on attempts to improve their microbicidal activity by substitution or modification of amino acid residues, while simultaneously enhancing their selectivity for prokaryote cells. In the case of tritrpticin and its C-terminally amidated version Tritrp1, the Trp, Arg and Pro residues have been shown to be important for the antimicrobial activity [16–21]. Mutated versions of Tritrp1 with Tyr and Phe residues replacing all three Trp revealed only small changes in microbicidal activity [16,18]. A similar result was obtained in this study, indicating that the addition of a hydroxyl group to the indole ring on all three Trp residues of Tritrp1 did not alter the peptide concentration required to kill *E. coli* cells. However MIC and MBC experiments cannot account directly for any changes in the mechanism of action that may accompany these modifications of the indole rings.

The bacterial membrane is one of the first barriers that govern the action of any AMP. Hence our work focused mostly on studying the interactions between the Tritrp1-derived peptides and different model membranes. Clearly these interactions are dominated by electrostatic interactions, as indicated by the preference for model membranes with negatively charged rather than neutral zwitterionic phospholipids headgroups. This behavior was also reflected by the larger fluorescence blue shifts and the smaller Ksv when binding to ePC:ePG in comparison to ePC:Chol vesicles, for the triple substituted (HW) peptide and Tritrp1. In spite of being less sensitive to the polarity of the environment [35,51], the 5OHW substituted peptide could still be used to confirm the

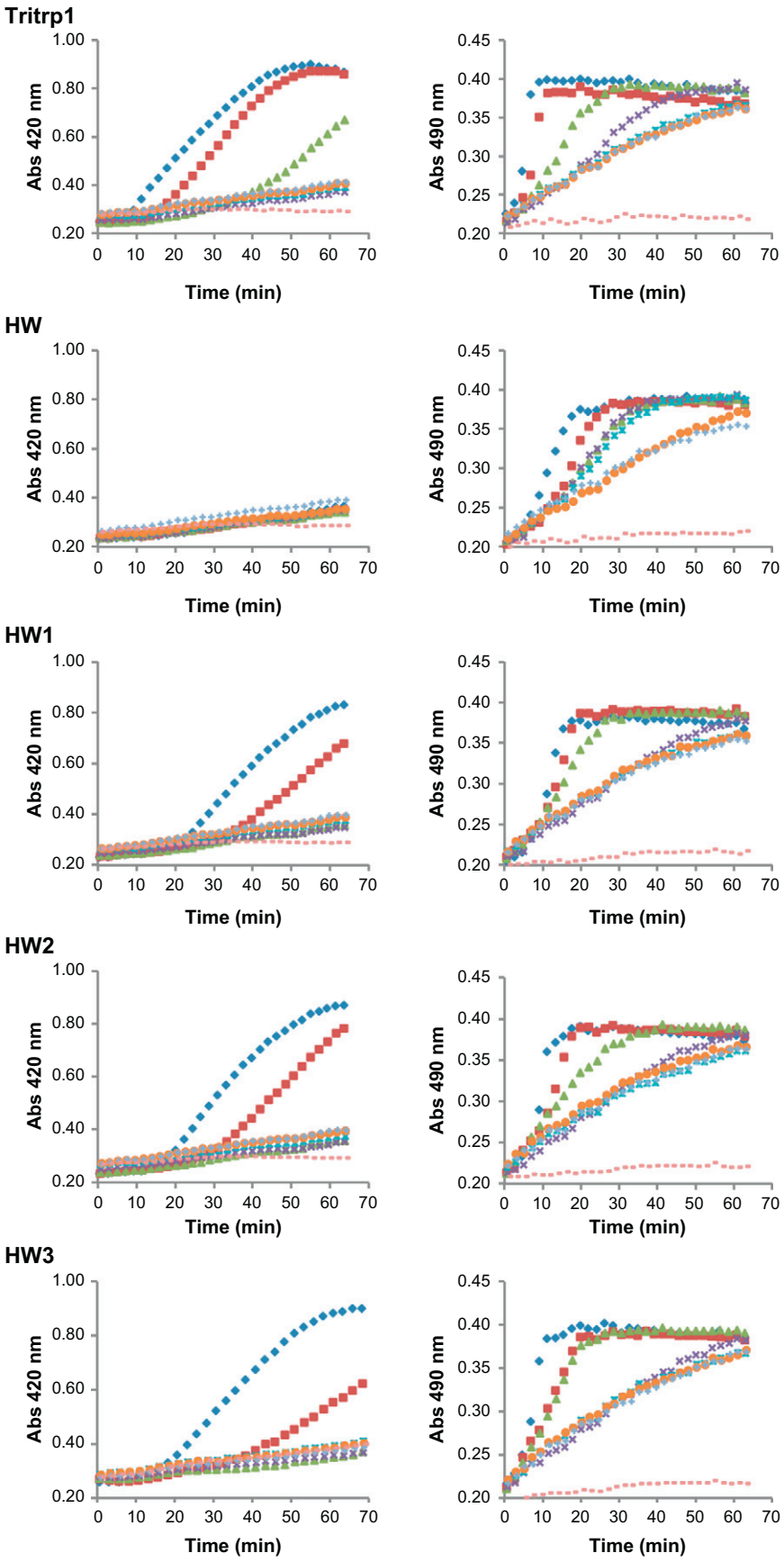

Fig. 7. Permeabilization of inner (*left*) and outer (*right*) membranes of *E. coli* ML 35p induced by the Tritrp1-derived peptides. Absorbance at 420 and 490 nm registered ONPG and NCF hydrolysis at 37 °C, respectively. Peptide concentrations for Tritrp1, HW1, HW2 and HW3 were as follows: 8.0 (◆), 4.0 (■), 2.0 (▲), 0.8 (●), 0.4 (×), 0.2 (+) and 0 μM (✳). HW concentrations were as follows: 20 (◆), 8.0 (■), 4.0 (▲), 2.0 (●), 0.8 (×), 0.4 (+) and 0 μM (✳). A control without cells (-) is also depicted. The experiments were performed in triplicate, representative results are shown.

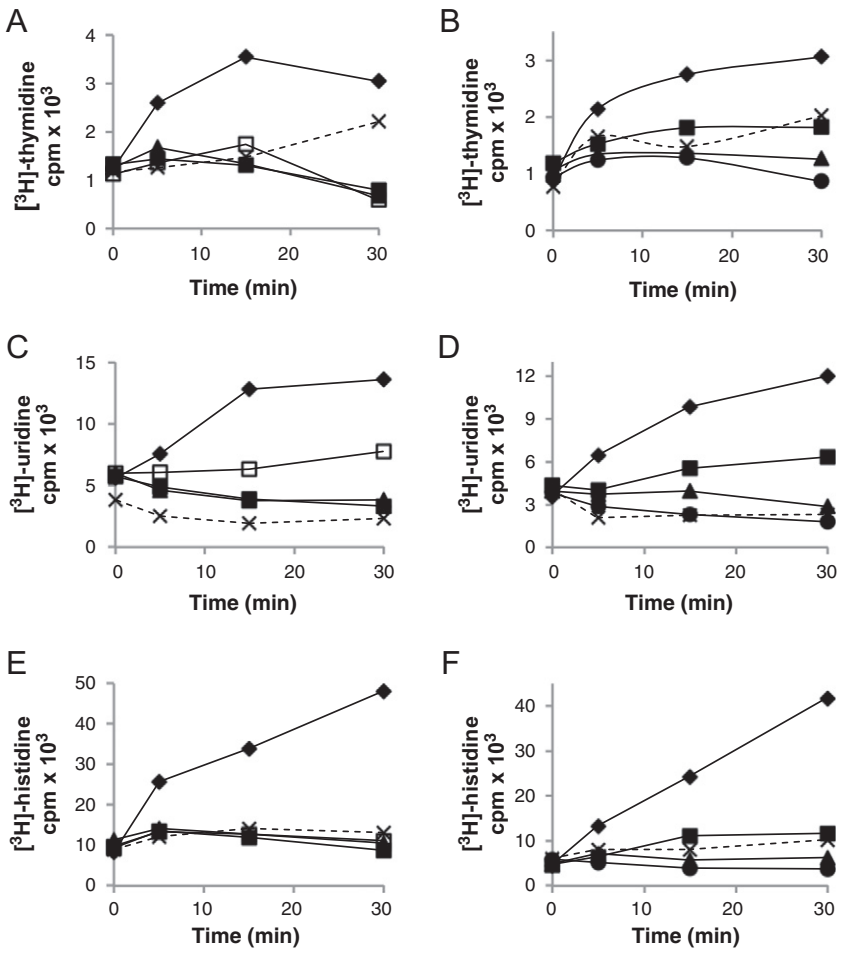

Fig. 8. Inhibition of the macromolecular synthesis of DNA (A, B), RNA (C, D) and proteins (E, F) induced Tritrp1 (*left*) and HW (*right*). Peptide concentrations were as follows: 8.0 (●), 4.0 (▲), 2.0 (■), 0.8 (□) and 0 μM (♦). The antibiotics (×) ciprofloxacin 1 μg/ml, rifampin 32 μg/ml and chloramphenicol 16 μg/ml were used as reference for DNA, RNA and protein synthesis inhibition, respectively. The experiments were performed in duplicate, representative results are shown.

interaction between HW and membranes (Table 3). However for all three individually substituted peptides (HW1, HW2 and HW3) the combined fluorescence maximum for Trp and 5OHW in buffer was close to 340 nm, indicating a more hydrophobic environment for the Trp residues. This characteristic of HW1, HW2 and HW3 precluded the use of blue shift as a reliable indicator of peptide-membrane interactions. Nevertheless acrylamide quenching experiments were successful in confirming the preference for negatively charged membranes for the bound peptides (Fig. 1). In addition to the fluorescence spectroscopy results, our DSC measurements also indicated a strong perturbation of the DPPG lipid packing induced by the interaction with all the 5OHW-containing peptides. Such perturbations were minimal in the presence of zwitterionic DPPC membranes (Fig. 5). A similar preference for model membranes with negatively charged phospholipid headgroups

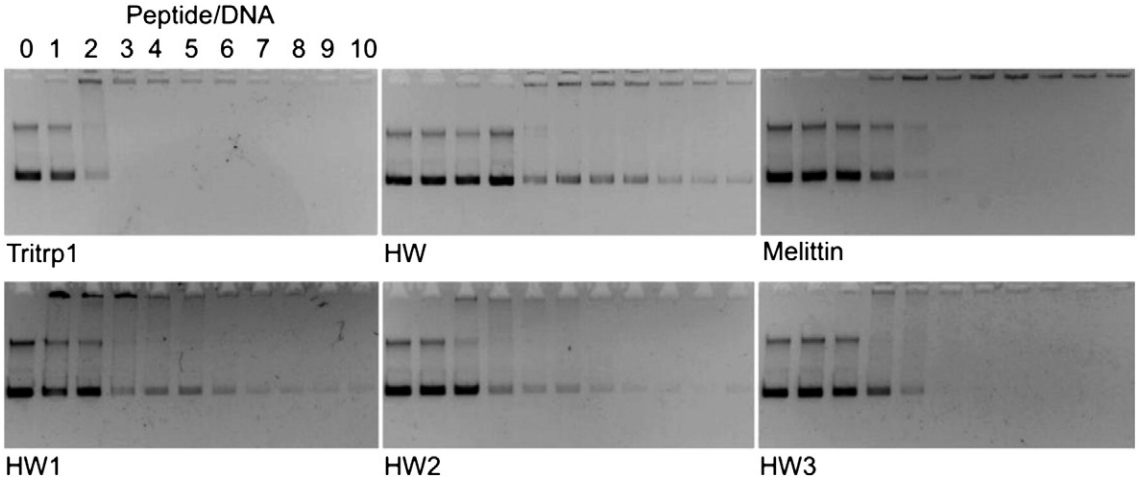

Fig. 9. Binding of Tritrp1-derived peptides and melittin to plasmid DNA (pET-19b) evaluated the by the gel retardation assay. Peptide to DNA ratios (w/w) between 0–10 were used. The experiments were performed in triplicate, representative results are shown.

was also exhibited by tritrpticin and several analogs when binding to DMPC and DMPG vesicles [54].

The binding of AMPs to membranes usually induces a conformational change in the peptide, which is believed to be important for their antimicrobial activity [55,56]. In this work all the single substituted peptides (HW1, HW2 and HW3) acquired different conformations upon binding to SDS micelles, as indicated by changes in their CD profile (Fig. 2). However the assignment of a specific secondary structure to these spectra is limited by the high content of aromatic residues that contribute to a strong band close to 230 nm. This band has been attributed to the interaction of Trp residues with the backbone of the peptide and stacking of these residues [57]. Furthermore the possibility of a heterogeneous population of conformers also restricts the interpretation of the CD spectra, providing only an average of all the different conformers co-existing in a particular case. Surprisingly, the CD spectra of the HW peptide did not undergo considerable changes when adding SDS micelles, suggesting that the structure of the peptide is not highly modified in this environment, or that it does not bind tightly to these micelles.

To obtain more detailed information about the possibility of multiple bound peptide conformations, 1D ^{1}H NMR spectroscopy was used (Fig. 3). The presence of the Trp and 5OHW residues allowed inspection of the spectral region between 11.0 and 9.0 ppm, where only the Hε1 protons of the indole rings are detected. This portion of the ^{1}H NMR spectra is far removed from the highly crowded 9.0–5.0 ppm region, where most of the aromatic and amide proton resonances are located. In all 5OHW containing peptides, except for HW, the change from an aqueous environment to the SDS micelles was accompanied by a reduction from several structural conformers to one major conformation. Similar results were previously reported for tritrpticin and some of its analogs [16,47]. The presence of multiple structural conformers is likely related to the *cis-trans* isomerization of the two Pro residues in the parent peptide [16,17]. Interestingly again, the HW peptide displayed a unique behavior as it exhibited one main conformation in aqueous environment as well as in the presence of SDS micelles. This phenomenon might be explained by inter- and/or intra-molecular interactions among the 5OHW residues, stabilizing the peptide's structure. Inter-molecular interactions would generate peptide dimers or oligomers, while intra-molecular interactions would create a more stable/structured peptide. The notion that such interactions occur for HW is also supported by the low efficiency of acrylamide quenching and the extraordinary red shifted fluorescence of the HW peptide ($\lambda_{em,\ max}$ 398 nm) observed in aqueous environment.[1]

In order to establish the detailed 3D structures of the peptides when bound to micelles, 2D ^{1}H NMR experiments were performed. However only the single substituted peptides could be analyzed and their structure calculated. The NMR spectra for HW proved to be very challenging due to low peak intensities and peak overlap, precluding the satisfactory assignment of the NMR resonances. HW1 preserved the turn-turn structure that is characteristic for its parent peptide tritrpticin bound to SDS micelles [17], and for Tritrp1 associated with DPC micelles [16]. In contrast, HW2 and HW3 diverged from this structural arrangement, exhibiting less extended structures with a more compact distribution of their hydrophobic residues. HW2 conserved one of the β-turns involving Pro-5, while HW3 did not exhibit any obvious β-turns. These changes allowed for a closer proximity between the N- and C-terminal regions of the peptides, generating a more compact amphipathic molecule. The formation of these amphipathic structures is in agreement with the strong interactions between the peptides and model membranes revealed by the previously discussed biophysical techniques. We note with interest that in spite of the different micelle-bound 3D-structures, these three peptides display essentially the same antimicrobial activity, indicating that the differences in the backbone conformation per se do not dictate the activity. Rather a more general amphipathic structural fold seems to be mainly responsible [5].

The mechanism of action of Tritrp1 has been related to its ability to induce membrane permeabilization, as observed by calcein leakage in different membrane mimetic systems [16,21]. In this study the three single substituted Tritrp1-derived peptides were also able to induce calcein leakage from zwitterionic and negatively charged vesicles. Interestingly, the Trp indole ring at position six was most important for promoting leakage by Tritrp1 in ePC:ePG vesicles, as shown by the decrease in calcein leakage in HW1. Calcein leakage was not observed upon modification of all three Trp residues in the HW peptide. These results are in agreement with the lack of permeabilization induced by Tritrp1 analogs were all Trp residues were replaced by Tyr or Phe residues [16,18]. Although these results suggest that membrane permeabilization might no longer be the main mechanism of action of the HW peptide, the fact that a very simple membrane mimetic LUV system was used precludes the confident extrapolation of this notion to the case of bacterial membranes [58].

In order to evaluate permeabilization in a more realistic in-vivo setting, actual bacterial cells (*E. coli*) were used. One important aspect of this experiment [46], is that membrane permeabilization can be divided into two steps, perturbation of the outer and inner membranes. The outer membrane is sometimes overlooked in mechanistic studies of AMPs, when only model membrane systems are used. However in several studies this barrier has been shown to modify the susceptibility of Gram-negative bacteria to some AMPs [58–60]. The inner membrane permeabilization experiments revealed a consistent behavior with the calcein leakage experiments. Tritrp1 and the single substituted peptides were able to induce inner membrane permeabilization, while HW was unable to permeabilize the cytoplasmic membrane of *E. coli* even at a concentration of 5× MIC. Interestingly, the outer membrane revealed a different behavior. As expected all peptides that induced inner membrane permeabilization also induced outer membrane permeabilization. Nevertheless, the inner membrane-inactive HW peptide could induce an increase in outer membrane permeabilization similar to Tritrp1 and the other analogs studied here. These results identified the outer membrane of *E. coli* as a main target for HW. At the same time we can conclude that Tritrp1's antimicrobial activity does not arise only from the perturbation of the inner membrane but also from disruptions of the *E. coli* outer membrane.

The strong ability of Tritrp1 to permeabilize the *E. coli* cytoplasmic membrane likely induces a complete collapse of many bacterial functions, due to the destruction of the electro-chemical gradient across the membrane. In this scenario, energy-dependent processes such as DNA, RNA and protein synthesis would also be completely inhibited. In this work Tritrp1 simultaneously inhibited the incorporation of radioactively labeled precursors for DNA, RNA and protein, when *E. coli* cells were exposed to sub-lethal concentrations of the peptide. In addition the strong affinity of Tritrp1 for plasmid DNA (Fig. 9), indicated that if Tritrp1 is able to reach the cytoplasm of the bacteria, following the permeabilization of the inner membrane, the interaction with the chromosomal DNA would likely directly inhibit the macromolecular synthesis as well.

HW on the other hand was also capable of simultaneously inhibiting the synthesis of DNA, RNA and proteins, although the process was much slower and higher peptide concentrations were required (Fig. 8). In combination with the lack of inner membrane permeabilization, these results suggest that the substitution of all three Trp residues by 5OHW in Tritrp1 modified the mechanism of action of the peptide. It suggest that HW, similar to Tritrp1, can gain access to the periplasm through the "self-promoted uptake" mechanism [61]. However, once in contact with the inner membrane a non-permeabilizing membrane translocation process might take place, releasing HW into the cytoplasm where it encounters different intracellular targets. HW can indeed bind to plasmid DNA and partially inhibit its migration through the agarose gel, suggesting that DNA could be one of HW's intracellular targets.

[1] In our laboratory similar red shifted fluorescence maxima have been observed in other Trp-rich antimicrobial peptides that were substituted with multiple 5OHW.

Nevertheless, it is important to stress that the inter- or intra-molecular interactions of HW in solution will likely modify its interactions with other peptide, membranes and DNA. Be that as it may our data shows that multiple 5OHW substitutions had a profound effect on the mechanism of action of Tritrp1.

5. Conclusions

In conclusion it was possible to identify that changes in the peptide–lipid interactions were induced by the substitution of all Trp residues by 5OHW in Tritrp1. These changes led to a different mechanism of antimicrobial activity in the case of Gram-negative bacteria, which did not involve the permeabilization of the cytoplasmic membrane. The interaction and perturbation of the outer membrane was the only membrane-related effect observed for the HW peptide. The antimicrobial activity of HW could be the result of direct perturbation of the outer membrane leading to cell arrest and consequently to death. However it is also possible that the peptide, similar to buforin II [62], translocates through the cytoplasmic membrane without affecting the membrane permeability. Once in the cytoplasm, interactions with intracellular targets would then inhibit the synthesis of macromolecules (DNA, RNA and protein), resulting in bacteria death. Additionally in our work the importance of the cluster of three Trp residues in the tritrpticin core was confirmed. Moreover the use of 5OHW as Trp substitutes proved to be useful in order to study the mechanism of action of Trp-rich AMPs. Furthermore we have demonstrated that 5OHW can also be used as a new amino acid analog to modulate the antimicrobial activity and mechanism of action of other Trp-rich antimicrobial peptides.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.08.024.

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