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Membrane interaction and secondary structure of *de novo* designed arginine-and tryptophan peptides with dual function

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ABSTRACT

Cell-penetrating peptides and antimicrobial peptides are two classes of positively charged membrane active peptides with several properties in common. The challenge is to combine knowledge about the membrane interaction mechanisms and structural properties of the two classes to design peptides with membrane-specific actions, useful either as transporters of cargo or as antibacterial substances. Membrane active peptides are commonly rich in arginine and tryptophan. We have previously designed a series of arg/trp peptides and investigated how the position and number of tryptophans affect cellular uptake. Here we explore the antimicrobial properties and the interaction with lipid model membranes of these peptides, using minimal inhibitory concentrations assay (MIC), circular dichroism (CD) and linear dichroism (LD). The results show that the arg/trp peptides inhibit the growth of the two gram positive strains *Staphylococcus aureus* and *Staphylococcus pyogenes*, with some individual variations depending on the position of the tryptophans. No inhibition of the gram negative strains *Proteus mirabilis* or *Pseudomonas aeruginosa* was noticed. CD indicated that when bound to lipid vesicles one of the peptides forms an α-helical like structure, whereas the other five exhibited rather random coiled structures. LD indicated that all six peptides were somehow aligned parallel with the membrane surface. Our results do not reveal any obvious connection between membrane interaction and antimicrobial effect for the studied peptides. By contrast cell-penetrating properties can be coupled to both the secondary structure and the degree of order of the peptides.

1. Introduction

Cell-penetrating peptides (CPPs) and antimicrobial peptides (AMPs) belong to the same group of membrane active peptides (MAPs). CPPs are positively charged peptides with the ability to cross cell membranes and deliver macromolecular cargo. AMPs are positively charged peptides with the ability to kill or inhibit the growth of bacteria. Because of their different functions, the fields of CPPs and AMPs have until quite recently been separated, but light has now been shed upon the strong resemblance between these peptide classes [1,2]. Several CPPs, including penetratin [3], pVEC [3,4], TP10 [4] and Tat [5], have been shown to have antimicrobial properties. Likewise AMPs, for example Magainin [6], Lactoferrin [7] and LL-37 [8] have been shown to be cell-penetrating.

MAPs can be either cell-penetrating or bactericidal depending on the lipid composition of the target plasma membrane [9] and on the amino acid sequence of the MAP [10–12]. Therefore, one way to distinguish between CPPs and AMPs could be to look at cell selectivity and therapeutic index for different cell types and peptides. By changing the peptide sequence it would then be possible to fine tune the function in accordance with the target cell membrane. Pep-1, for example, is a very effective CPP with a moderate antibacterial efficiency. By modifying the Pep-1 amino acid sequence with Glu to Lys substitutions, derivates have been made that were both highly bactericidal and cell selective [13,14]. Also, the insertion of one or several tryptophan moieties into peptide sequences has been shown to affect both the cell-penetrating efficiency [15,16] as well as the anti bacterial effect [17–19]. Tryptophans are, together with arginines, often found in naturally occurring CPPs and AMPs [20,21] and short synthetic peptides rich in these two amino acids have been proven effective as both transporters and antibacterial agents [15,22,23]. However, systematic investigations of how tryptophans affect the therapeutic index of tryptophan/arginine rich peptides are still scarce.

To date, the effect of tryptophans on antimicrobial activity has been mostly studied in the form of end-tagging and single moiety insertions. We have previously shown that the number and position of tryptophan in the amino acid sequence affect cell internalization efficiency of CPPs consisting of eight arginines and one to four tryptophans at different positions [16]. In the light of the strong resemblance found between CPPs and AMPs, we investigate whether our *de novo* designed CPPs (Table 1) also have antimicrobial properties. We have also evaluated if the tryptophan number

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Table 1
Peptide sequences, uptake efficiency and cytotoxicity.

Peptide	Sequence	Charge	Hydrophobicresidues	Uptake efficiency CHO-cells[a]	Cytotoxicity CHO-cells[b]
WR_8	WRRRRRRRR	+8	1	+	-
W_2R_8	WWRRRRRRRR	+8	2	+	-
W_3R_8	WWWRRRRRRRR	+8	3	+	+
W_4R_8	WWWWRRRRRRRR	+8	4	+	+++
RWR	RRRRWWWRRRR	+8	4	++	+
RWmix	RWRRWRRWRRWR	+8	4	+++	+
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	+5	16	No data	No data

[a] Uptake efficiency of 5-FAM labeled peptide in live CHO-cells (Chinese Hamster Ovarian cells) measured with flow cytometry [16].
[b] Peptide induced cytotoxicity of CHO-cells measured with flow cytometry [16].

and position affect the membrane interaction and induction of secondary structure upon binding, and if variations in such interactions would be reflected in the antimicrobial function.

2. Materials and methods

2.1. Chemicals

The RW-peptides (>95% purity) were purchased from Innovagen (Lund, Sweden). Melittin was purchased from Sigma–Aldrich (Stockholm, Sweden). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) were purchased from Larodan Fine Chemicals (Malmö, Sweden).

2.2. Minimal inhibitory concentration

Minimal inhibitory concentration (MIC) was measured using standard procedure. Four bacteria strains were used: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Staphylococcus pyogenes*. Bacteria colonies were picked from a fresh (18 h to 24 h) agar plate and dispensed in 0.9% NaCl followed by vortexing. The bacteria concentration was matched to McFarland standard 0.5 (1.5×10^8 CFU/ml), whereafter the bacteria suspension was diluted in water supplemented with 0.02% Tween 80 to final concentration of 1×10^5–5×10^5 CFU/ml. A micro dilution tray was prepared for each bacterial strain, with cation-adjusted Mueller–Hinton broth (CAMHB) and the peptides (64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 μg/ml). 0.01 ml of bacteria suspension was added to each well of the micro dilution tray, followed by incubation for 24 h at 35 °C. As negative control CAMHB only was used. Also, purity plates were inoculated for each bacteria strain. The bacterial growth was determined measuring OD at 600 nm.

2.3. Liposome preparation

POPC and POPG, dissolved in chloroform, were mixed in a round bottom flask to a molar ratio of 80/20. The solvent was evaporated under reduced pressure using a rotary evaporator. The remaining lipid film was placed under vacuum over night to remove any solvent traces. The liposomes were formed by vortexing the lipid film with 10 mM phosphate buffer (supplemented with sucrose for LD measurements) for 5 min, followed by five freeze-thaw cycles (liquid nitrogen/50 °C) and extrusion 21 times through Nucleopore polycarbonate filters with pore diameter of 100 nm using an extruder (LiposoFast-Pneumatic, Avestin, Canada).

2.4. Circular dichroism spectroscopy

Circular dichroism (CD) [24] was used to study the secondary structure of the peptides in phosphate buffer (10 mM, pH 7.4) and when bound to LUVs. Spectra were recorded between 185 nm and 270 nm on a Chirascan Circular Dichroism Spectrometer (Applied Photophysics, UK) and the time per point was 0.500 s. The samples were measured at 20 °C in a 2 mm pathlength quartz cell. For each sample 20 scans were recorded and averaged. Spectra were corrected for background contributions by subtraction of appropriate blanks. The peptide concentration was 5 μM and the peptide-to-lipid molar ratio was 1:100. The peptide secondary structure was evaluated by comparison with standard reference spectra [25,26].

2.5. Linear dichroism spectroscopy

Linear dichroism (LD) is defined as the differential absorption of linearly polarized light parallel and perpendicular to an orientation axis [24]. In our liposome system the unique axis is the membrane normal. The transition moment related to a positive LD signal in this system is perpendicular to the normal and hence parallel to the liposome surface and vice versa for a negative signal [27]. By normalizing the LD spectrum with respect to the isotropic absorption (A_{iso}), the concentration- and pathlength-independent quantity reduced linear dichroism (LD^r) is obtained. For the LD measurements a Chirascan Circular Dichroism Spectrometer fitted with a Linear Dichroism detector (Applied Photophysics, UK) was used. The samples were oriented by shear flow using an outer-rotating quartz Couette cell with a light path of 1 mm under a shear flow of 3100 s^{-1}. Spectra were corrected for background contributions by subtracting the corresponding spectrum without rotation of the Couette cell (isotropic sample). Isotropic absorption measurements on all samples were made with a Varian Cary Bio 50 (Agilent Technologies, USA) using a 4 mm pathlength quartz cuvette. In order to reduce light scattering from the LUVs and to improve the macroscopic orientation [28], 50% sucrose by weight was used in the buffer in all LD measurements. 24 μM of the peptide was mixed with LUVs to a peptide-to-lipid ratio of 1:100.

3. Results

3.1. MIC

To investigate whether the RW-peptides are bactericidal, MIC (minimum inhibitory concentration) measurements were performed. Four bacterial strains, two gram positive (*S. aureus* and *S. pyogenes*) and two gram negative (*P. aeruginosa* and *P. mirabilis*), were assessed. The RW-peptides inhibit bacterial growth of the two gram positive strains at levels similar (*S. aureus*) and somewhat superior (*S. pyogenes*) to melittin (Table 2). The MIC values for melittin are in accordance with previous results [29]. For *S. aureus*, W_4R_8 and RWR both show a minimal inhibitory concentration of around 4 μM, and W_3R_8, W_2R_8 and RWmix all show MICs of between 5 μM and 6 μM. For *S. pyogenes* W_4R_8 is evidently superior to the other peptides at inhibiting the bacterial growth, with MIC values almost