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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

Table 2
MIC values in μg/ml and μM of the tryptophan/arginine peptides and melittin.

	S. aureus		*P. aeruginosa*		*P. mirabilis*		*S. pyogenes*	
MIC*	μg/ml	μM	μg/ml	μM	μg/ml	μM	μg/ml	μM
WR_8	32	21	>64	>43	>64	>43	4	2.7
W_2R_8	9	5.4	>64	>38	>64	>38	1	0.59
W_3R_8	10	5.4	>64	>34	>64	>34	0.875	0.47
W_4R_8	9	4.4	>64	>31	>64	>31	0.25	0.12
RWR	8	3.9	>64	>31	>64	>31	1.625	0.79
RWmix	12	5.8	>64	>31	>64	>31	1	0.49
Melittin	16	5.6	64	22	>64	>22	4	1.4

* MIC, minimal inhibitory concentration required for total growth inhibition in liquid medium. The values represent mean values from two (*S. aureus* and *S. pyogenes*) or one (*P. aeruginosa* and *P. mirabilis*) independent experiment(s) with four replicates each. Complete MIC results are found in Supplementary material.

10 times lower than for melittin. For both of the gram positive strains, the WR_8 peptide is evidently less potent than the other five peptides. The RW-peptides do not inhibit the bacterial growth of the two gram negative bacteria *P. aeruginosa* and *P. mirabilis* within the concentration regime tested, while melittin shows MIC values of 22 μM approximately as expected [29,30].

3.2. Circular dichroism

Circular dichroism measurements were performed in order to reveal differences in secondary structure between the peptides in solution and when interacting with the lipid membranes of liposomes. In Fig. 1B, a CD spectrum is seen consistent with RWmix adopting an α-helical like structure, but not a perfect helix, when interacting with liposomes, with positive maximum below 200 nm and negative troughs at around 205 nm and 222 nm [24]. In buffer RWmix has a random-coil structure. WR_8 shows a random-coil like structure, both in solution as well as when bound to liposomes (Fig. 1G). The other RW-peptides mainly showed CD spectra that were assigned as random coil structures, but with the addition of a negative trough at 228 nm, both when bound and when free in solution (Fig. 1C–F). Melittin was, as previously shown by others [31–33], adopting an evident helical structure when binding to the lipid membrane and random-coil in solution (Fig. 1A).

3.3. Linear dichroism

To further examine the peptide-membrane interactions flow LD spectroscopy applied to shear-deformed liposomes was performed. The peptide backbone absorption in the far-UV can be used to determine the orientation of the peptide relative to a lipid surface. Because of exciton coupling, the π–π* transition moments of the neighboring peptide bonds will in an α-helix split into two transition moments, one parallel to the helix (200 nm to 210 nm) and one perpendicular to the helix (<200 nm). Further, the LD signal from the aromatic tryptophan residues can give additional information about their orientation. The indole of tryptophan has three transition moments in this region, B_b at 215 nm to 240 nm, L_a at around 270 nm and L_b at around 290 nm [34,35]. Fig. 2 shows the LD (A) and LD^r (B) for peptides in presence of 80/20 mol% POPC/POPG liposomes. For simplicity only the three RW-peptides containing four tryptophans are shown. The LD^r signals for RWmix are relatively strong, compared to the other peptides, meaning that this peptide thus has a better orientation than the other peptides, both regarding allowed electric dipole transitions of the α-helix structure (around 209 nm for the long axis polarized transition) and the transition moments of tryptophan (around 225 nm, 270 nm and 290 nm). RWR shows an intermediate LD signal, whereas the signal for W_4R_8 is very small suggesting a poor orientation. The other peptides, WR_8, W_2R_8, W_3R_8 and melittin show LD signals of the same spectral shape and with LD^r amplitudes between those of RWR and W_4R_8. The LD^r spectra also show that the absorption peak at 209 nm (α-helix long axis) appears to have a positive sign, meaning that the peptides should be oriented parallel rather than perpendicular relative to the membrane surface

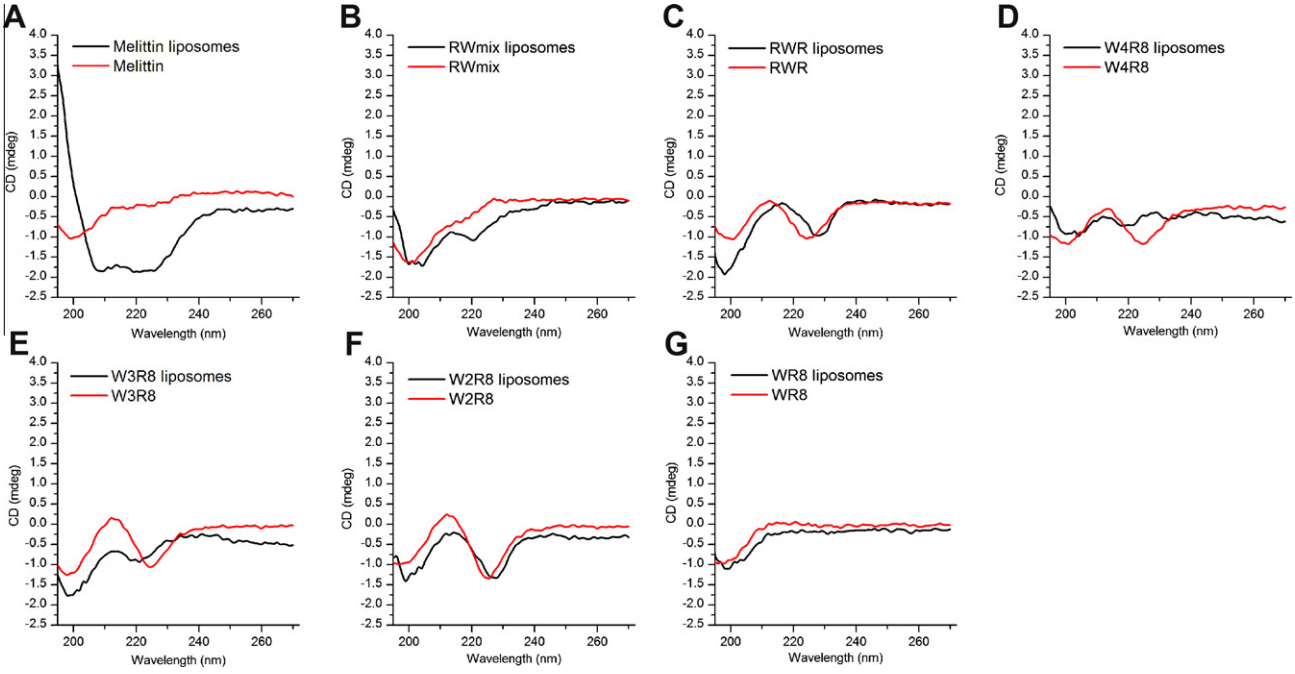

Fig. 1. Circular dichroism (CD) spectra of the RW-peptides free in solution (red) and when associated with POPC/POPG 80/20 mol% liposomes (black). The peptide concentration was 5 μM and the lipid concentration was 0.5 mM. The spectra presented are averages of 20 repeated scans, and each measurement was performed in two (melittin) or five (RW-peptides) replicates.

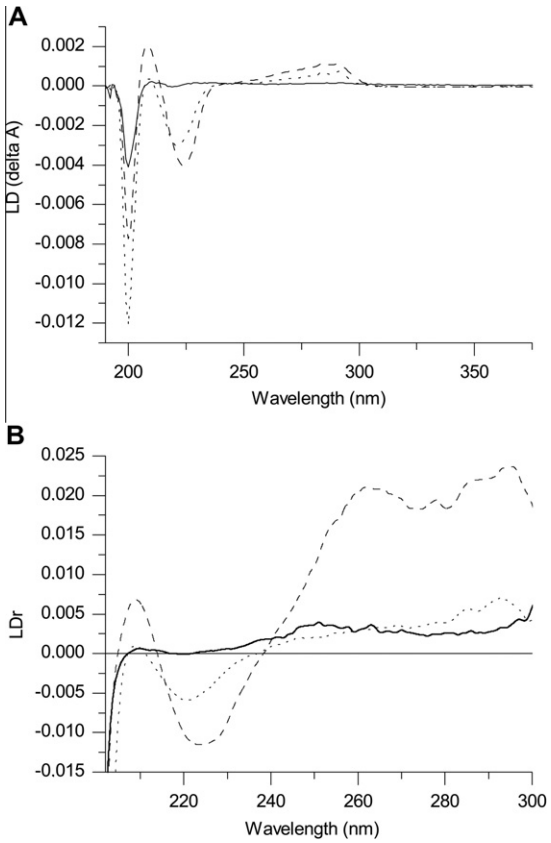

Fig. 2. Linear dichroism (LD) spectra (A) and reduced linear dichroism (LD^r) (B) of W_4R_8 (solid), RWmix (dashed) and RWR (dotted) when associated to liposomes consisting of 80/20 mol% POPC/POPG. The peptide concentration was 25 μM and the lipid concentration was 2.5 mM.

[36]. However, the signals at 209 nm are, with the exception of RWmix, very weak and the significance of this assignment of orientation, therefore, can be discussed.

4. Discussion

In a previous work, we have shown that uptake efficiency of six RW-peptides into mammalian cells was the highest when the tryptophans were positioned in the middle or scattered along the sequence [16]. Further, the uptake efficiency did not coincide directly with increased cytotoxicity and only one of the peptides, W_4R_8, was clearly toxic to mammalian cells. We hypothesized that the observed differences between the peptides might be caused by differences in membrane interactions and changes in secondary structure upon membrane binding. In the light of the high resemblance between CPPs and AMPs, we wanted to examine if the peptides might also be antibacterial.

The combination of charges and hydrophobicity is considered to be the basis of the antimicrobial effect. AMPs normally have a net charge of +4 to +6 and up to 50% of the amino acid residues are hydrophobic [2]. When exploring our MIC results, it is suggested that the position more than the number of tryptophans determine the effect against *S. aureus* and *S. pyogenes*. For *S. aureus*, W_4R_8 and RWR both show minimal inhibitory concentrations of around 4 μM, whereas the MICs for W_3R_8, W_2R_8 and RWmix are somewhat higher. For *S. pyogenes*, W_4R_8 is evidently superior to the other peptides at inhibiting the bacterial growth. WR_8 is for both strains evidently inferior as antibacterial agent. These results suggest that in addition to the amount of hydrophobic residues in a sequence, the position may also affect the antimicrobial function. The effect tends to be higher when the hydrophobic tryptophan resides are placed next to each other, and even higher when the accumulation of tryptophans is placed at the N-terminal end of the sequence. A possible explanation could be that when separating the hydrophobic and charged residues, the amphipathicity becomes pronounced, rendering a more membrane active peptide. The two gram negative bacteria strains investigated in this work where not inhibited by the peptides in the concentration regime tested. This could hypothetically be explained by that interaction with the thick outer peptidoglycan layer of the gram positive bacteria, possibly resulting in increased local peptide concentration at the surface, is needed for efficient antibacterial effect.

When looking at the induction of secondary structure, it is seen that the RW-peptides interact differently with the lipid membrane. RWmix adopts a structure with elements similar to that of an α-helix. The weak helical signal may be caused by the short peptide length. WR_8 shows the lowest CD signal with a vague random coil-like structure. The other four RW-peptides also show random coil structures, but with the addition of negative peaks at around 228 nm, which are very probably interactions between adjacent tryptophan residues [18]. When bound to liposomes, the signal for W_4R_8 is unfortunately hard to evaluate, possibly because of aggregation of the lipid vesicles.

From linear dichroism we receive further information about the peptides' interactions with lipid surfaces. In accordance with the CD results, the higher orientation of RWmix, both of the tryptophans and the long helical axis, points to the possibility that this peptide has a partly α-helical structure with the tryptophans being oriented in the same direction. Since it takes slightly more than 3 amino acids for one turn in a helical wheel, it is thus only when the tryptophans are positioned at every third position, as in RWmix, that they can be fully oriented. For the other peptides, the number of tryptophans and/or the lack of tryptophan orientation might cause the weak LD signals observed for the tryptophan transition moments. The lower degree of orientation seen when the tryptophans are placed next to each other in the amino acid sequence, might be caused by steric hindrance. The results from the LD measurement also point to the possibility of the RW peptides being situated horizontally at the membrane, probably with one or more of the tryptophans inserted into the membrane.

Combining these observations, no clear connection between secondary structure and bacterial growth inhibition efficiency could be monitored. RWmix has the most pronounced secondary structure and the highest degree of orientation, but is not the most prominent antibacterial agent in the group. The antibacterial effect seems instead to depend on the amphipathicity. In contrast, the secondary structure and degree of orientation seem to be directly connected to the cell-penetrating properties, with RWmix and RWR having the highest degree of orientation and being superior CPPs (Table 1). Regarding therapeutic index, W_4R_8 is the best antibacterial agent but it is also toxic to mammalian cells. The peptide that has the best ratio is W_2R_8, which is not more toxic than buffer (Table 1) and fairly effective as AMP. W_2R_8 also has lower internalization efficiency into cells in comparison with the other RW-peptides. These results show that the position of the tryptophan residues in a sequence may regulate the therapeutic index and tuning the peptide function between CPP and AMP, properties that may be useful in the design of better CPPs and AMPs in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.030.

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