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Biological consequences of improving the structural stability of hairpins that have antimicrobial activity

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Abstract

Designing new antimicrobial peptides (AMPs) focuses heavily on the activity of the peptide and less on the elements that stabilize the secondary structure of these peptides. Studies have shown that improving the structure of naturally occurring AMPs can affect activity and so here we explore the relationship between structure and activity of two non-naturally occurring AMPs. We have used a backbone-cyclized peptide as a template and designed an uncyclized analogue of this peptide that has antimicrobial activity. However, improvements to the structure of this peptide reduced the activity of the peptide against gram-negative, *E.coli* but improved the activity against gram-positive, *C.glu*. The disagreement between gram-negative and gram-positive activity was also seen in a second peptide system. Structural improvements resulted in a peptide that was more active than the native against gram-positive bacterium but less active against gram-negative bacterium. Our results show that there is not always a correlation between structure and activity. Other factors such as the type of bacteria being targeted as well as net positive charge can play a role in the potency of AMPs.

Keywords

Antimicrobial; hairpin peptides; structure; activity

Introduction

Naturally occurring antimicrobial peptides (AMPs) have generated much interest in the effort to find solutions to combat multiple drug resistant bacteria. AMPs are typically small, less than 50 amino acids, with a high number of cationic residues as well as hydrophobic residues that impart an amphipathic character to the peptide. They adopt a variety of secondary structures such as α -helix, β -hairpins and linear or loop conformations. [1–4] AMPs have been extensively studied in efforts to improve their activity as well as developing the ability to rationally design new AMPs with better AMP activity but lower hemolytic activity. [5,6]

Protegrin-I (PG1) is a β -hairpin AMP that is found in porcine leukocytes. NMR studies confirm that the presence of two disulfide bridges constrain the peptide to a well-defined β -

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hairpin conformation in solution. The relationship between the activity and the structure has been studied by removing the one or both disulfide bridges. The resulting peptide lacks the defined secondary structure of the native sequence resulting in a loss of activity. [7] This has led to the hypothesis that increased hairpin structuring could lead to the development of new AMPs that have equal or better activity than native peptides. To this end, multiple studies have been done on backbone cyclized peptide analogues of PG1, tachyplesin I (another β -hairpin AMP) as well as many others. [8–10]

Tam et al. have developed several analogues of both tachyplesin I and PG1 that are backbone cyclized and contain a varying number of disulfide bridges within the sequence in order to stabilize the β -hairpin conformation. Their analogues of tachyplesin I showed similar AMP activity as the native peptide but had a much lower hemolytic activity toward human erythrocytes. [8] In an additional study they applied a similar methodology to designing cyclic analogues of PG1 that displayed improved AMP activity against several gram-negative and gram-positive strains of bacteria as well as a large decrease in hemolytic activity. [9] These results demonstrate that improving the stability of peptide secondary structure can play a role in increasing the potency of an AMP.

In 2002, Shankaramma et al. developed a method for designing cyclic AMP mimetics using a D-Pro-L-Pro turn at one terminus. [10] In their study, the cationic sequence of several AMPs was grafted onto this template and tested for antimicrobial activity. Their screening yielded 7 mimetics that had activity against gram-positive and gram-negative bacteria. One species, mimetic 4, stood out as having reasonable AMP activity and extremely low hemolytic activity. These results led to a study by Robinson et al. whereby point variations were made to mimetic 4 using natural and unnatural amino acids to further improve the activity of the peptide. [11]

However, the disadvantage of this approach to stabilization is the difficulty in synthesizing cyclic peptides. Backbone cyclized peptides require multiple steps of complex synthesis and often result in lower yields. Further, the cyclization of a peptide results in an extreme rigidity being conferred that may limit the activity of the peptide; some flexibility may be required for receptor binding or activation. We therefore set out to discover if we could apply rational design in order to stabilize the β -hairpin structures of these peptides without having to depend on covalent linkages such as disulfide bridges and backbone cyclization. We have previously described variants to tachyplesin I that have no disulfide bridges and yet have demonstrated similar activity as native tachyplesin I against gram-positive and gram-negative bacteria. [12]

In this study, we extended these techniques to mimetic 4 described by Robinson et al. We have designed six peptides that do not rely on backbone cyclization or disulfide stabilization to form stable β -hairpin secondary structures. Instead we utilize an end capping motif, known as the B-cap, first described by Kier et al. This motif has been shown to increase the stability of an uncyclized hairpin peptide by approximately 6kJ/mol. The b-cap requires the use of a pair of trp residues at non-H-bonded positions at the termini. The trp/trp pair forms an edge-to-face interaction that can be monitored by CD. This motif is an important part of our structural optimization because it is a non-covalent interaction that has been shown to

have a higher stability than covalent interactions such as disulfide bonds. We also examine the effects of variation of residues within the strands of the hairpin on the stability of the peptide fold. Our variants allow us to study if the position of the positive residue is important to the structure and activity of our acyclin peptides.

We have also applied our rational design methods to another peptide, 1-RW. [13] This peptide, initially designed by Yu et al. is a completely novel peptide that was based upon a concept demonstrated by Frecer et al. In the study done by Frecer et al., synthesized peptides with the repeating amino acid sequence motif, cationic-hydrophobic-polar, had membrane binding properties. [14] These peptides contained alternating Val and Lys amino acids and had a disulfide bridge at the terminus to ensure that a β -hairpin conformation is adopted. Their peptides showed high affinity for binding to lipids which is a desirable trait for an AMP since the action of AMPs has largely been assumed to be membrane depolarization of target cells that results in rapid cell death. Yu et al. adopted this concept and designed several AMPs that contained the cationic-hydrophobic-polar amino acid motif along with a loop motif Gly-Ser-Gly in the middle of the peptide. [15] Their studies yielded several peptides with good AMP activity and low hemolytic activity. Their follow up study improved upon their AMP sequence by substituting all Phe and Lys residues with Trp and Arg residues, respectively. [13] This yielded peptide, 1-RW that had AMP activity as well as significant lipid binding. However, this peptide shows little or no ordered secondary structure. Since structure has been shown to play a part in the activity of AMPs, we applied rational design to this peptide to create analogues that have higher hairpin stability and increased AMP activity against gram-positive bacteria. Our modifications include optimization of the turn sequence in order to nucleate hairpin turn formation as well as addition of the b-cap to the termini to reduce fraying in this region.

Materials and Methods

Peptide synthesis and purification

All peptides were synthesized using standard Fmoc-solid phase peptide synthesis chemistry on a CEM Liberty Blue Microwave Synthesizer. 0.1 mmol resin was used and 5-fold excess amino acids were used for each coupling. N,N-diisopropylcarbodiimide (DIC) and oxyma were utilized as coupling reagents in 5-fold excess and 3 mL of 20% piperidine was used for deprotection. Peptides were cleaved in 9.5 mL trifluoroacetic acid, with 250 μ L triisopropylsilane and 250 μ L water used scavengers. Cleavage was allowed to occur over 90 minutes with mild shaking. Crude peptide was precipitated with cold diethyl ether.

Crude peptides were then purified using reverse phase HPLC. Peptides were dissolved in 5 to 7 ml of 50/50 water/acetonitrile and purified on a Varian Prostar 220 HPLC with an Agilent C18 column. A gradient of 10–60 % acetonitrile was used: eluent A was 1% TFA in water and eluent B was 0.085% TFA in acetonitrile. HPLC traces were monitored with UV detection at 220 nm and 280 nm wavelength. The collected fractions were lyophilized and stored as trifluoroacetate salts. Electrospray mass spectrometry (Bruker Esquire Ion Trap Mass Spectrometer) was used to analyze the purity of the peptide. A peptide purity at or above 97% was the criterion for further characterization.

Nuclear Magnetic Resonance experiments

Lyophilized peptides were dissolved in 90% 50mM phosphate pH 6.5 buffer with 10% 1mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) in D₂O. DSS served as the internal standard and D₂O was used as the lock solvent. The NMR sample was made to a concentration of 1mM by weight. NMR data was collected on Bruker AV700 and AV800 spectrometers.

Resonances were assigned to residues using the Sparky NMR assignment program with the usual 2D ¹H TOCSY and NOESY method to verify amino acid sequence as well as cross-strand interactions. Chemical shifts were then entered into our chemical shift database (CSDb) (<http://andersenlab.chem.washington.edu/CSDb>) with an in-house algorithm that corrects for neighboring inductive effects, temperature and pH differences, to calculate chemical shift deviations (CSDs).

The chemical shift deviation is given by the following equation:

$$CSD = \delta_{observed} - \delta_{known random coil value}$$

Where δ is the chemical shift in ppm.

Circular Dichroism Experiments

Lyophilized peptides were dissolved in 50 mM Phosphate pH 6.5 and concentration was measured using UV-Vis. The Beer's law equation was used to determine concentrations of stock solutions:

$$A = \epsilon cl$$

A = absorbance, ϵ = extinction coefficient, c = concentration and l = pathlength of the cell.

The following extinction coefficients were used: ϵ_{280} (Trp) = 5690 cm⁻¹M⁻¹; ϵ_{280} (Tyr) = 1280 cm⁻¹M⁻¹. For peptides with multiple Trp and Tyr residues, the extinction coefficient used was a sum of all the extinction coefficient of all the residues.

For CD studies (Jasco 720 Circular Dichroism Spectrometer with a 1mM pathlength cell), stock solutions were diluted to a final peptide concentration of 30 μ M (confirmed by UV-Vis). Each spectrum was an average of 8 scans taken between 190 to 270 nm. The following parameters were used: scan rate of 100 nm/min with a 2 nm bandwidth and a 0.1 nm step resolution. 10 spectra were gathered for each peptide between 5°C to 95°C at 10°C intervals. Spectra were then processed using Jasco Spectra Analysis software in which reverse Fourier transform was used to smoothen the spectrum and subtraction of the blank containing just buffer was done. The CD data is given as molar ellipticity (deg·cm²·dmol⁻¹) versus wavelength.

Antibacterial Assay

All peptide stock solutions were made with 50 mM Phosphate buffer pH 6.5. For gram-positive assays, sterile water was used to make buffers. The concentration of the stock solution was measured by UV-Vis as described above. To obtain a range of concentrations, stock solutions were serially diluted as needed.

Escherichia coli (Dh5α) were grown overnight at 37 °C in 5 mL sterile LB media. From the overnight culture 1 mL was removed and cells were pelleted. The cell pellet was resuspended in 5 mL fresh media and shaking at 37 °C was resumed. Growth was tracked by measuring the absorbance at 600 nm (OD₆₀₀) until the measured absorbance was between 0.5 and 0.8. The culture was diluted to an OD₆₀₀ of 0.001 from which 180 µL aliquots were taken and combined with 20 µL of peptide stock solution in a 96 well plate. Plate lids were sealed on three sides with parafilm to reduce evaporation. Peptide stock solutions with concentrations from 3.9 µM to 1 mM, obtained by serial dilution were used and each concentration was tested in triplicate. Control wells for 100% growth include 20 µL of water in place of the peptide solution; the background controls (no growth) consist of 200 µL of the LB broth alone. OD₆₀₀ is measured for each well after 16 h of incubation at 37 °C using a Thermo Labsystems Multiskan Spectrum UV/Visible microplate reader.

Corynebacterium glutamicum (ATCC 13032) were grown at 30 °C in sterile LBG media (LB with 0.5% sterile filtered glucose). Overnight cultures were pelleted by spinning for 5 min at 1500 r.p.m. and the pellet was resuspended in 1 mL fresh media. From the resuspended pellet 200 µL was used to inoculate 5 mL of sterile media, which was placed in a 30 °C shaking incubator. Growth was tracked until an OD₆₀₀ of > 0.25 was reached. The culture was serially diluted to an OD₆₀₀ of 0.001 from which 180 µL aliquots were taken and combined with 20 µL of peptide stock solution in a 96-well plate. Plate lids were sealed on three sides with parafilm to reduce evaporation. Peptide stock solutions with concentrations from 0.06 to 7.81 µM, obtained by serial dilution were used and each concentration was tested in triplicate. The control wells for 100% growth include 20 µL of water in place of the peptide solution; the background controls (no growth) consist of 200 µL of the LB broth alone. OD₆₀₀ is measured for each well after 16, 24, and 48 h of incubation at 37 °C using a Thermo Labsystems Multiskan Spectrum UV/Visible microplate reader.

The % growth of the bacteria for both assays was calculated using the following equation:

$$\% \text{ growth} = \frac{\text{Peptide}(OD_{600}) - \text{Background}(OD_{600})}{\text{Control}(OD_{600}) - \text{Background}(OD_{600})} \times 100$$

The minimum inhibitory concentration (MIC) where % growth was at or below 0% and the concentration of peptide required to achieve 50% growth (IC₅₀) were determined by observation of the data once plotted on a graph of % growth versus peptide concentration.

Results and Discussion

The original PG1 analogue being analyzed, mimetic 4, had the following sequence: cyc(-LRLKKRRWKYRVpP-). Based upon previous studies into non-covalent stabilizations, we eliminated the D-Pro-L-Pro turn and replaced it with what we designate as a β -cap. [16] The β -cap was described by Kier et al. and demonstrated a 6 kJ/mol increase in stability of the hairpin peptide. [16] This specific β -cap requires the presence of an alkanoyl-Trp sequence at the N-terminus and a Trp-Thr-Gly at the C-terminus. Capping results from the interaction of 2 cross strand Trp residues at the N and C-terminus of the peptide. A pair of Trp residues, at non-H bonded positions, are able to form an edge-to-face (EtF) interaction that can be observed by CD. [17] The presence of an EtF interaction is evident by CD when a bisignate curve with a minimum at 215 nm and a maximum at 228 nm is observed. For the purposes of this study, we focus on the maximum at 228 nm because the region between 205 nm and 220 nm is complicated by multiple secondary structural characteristics of hairpin peptides. The presence of a β -strand is typically observed by a minimum between 216 nm and 220 nm while the presence of a hairpin turn can also have contributions in this region. The presence of these other characteristics can mask the minimum at 215 nm that is due to the exciton couplet and lead to inaccurate interpretation. This is why we focus on the peak at 228 nm to monitor the EtF interaction. The decrease in intensity of this maxima as temperature is increased can be used to calculate a melting temperature and, thus, the stability of β -hairpin fold essential for β -cap formation.

The Trp/Trp EtF interaction has been shown to provide at least a 2.3–5 kJ/mol stabilization of hairpin folds. [16,18] More recently, we have reported that end-capping Trp/Trp EtF can produce a stabilization of 5–7 kJ/mol. [19] Some stabilization also results from the interaction of the Gly-H N interacting with the indole ring of the C-terminus Trp, and the Thr hydroxyl group interacting with the C=O of the Trp-alkanoyl peptide bond. The stability of this cap has been shown to be higher than that conferred by covalent stabilization such as disulfide bonds by ~2kJ/mol. The presence of this cap could thus serve as a replacement for the backbone cyclization of the peptide and since it is a non-covalent interaction, it allows the peptide to adopt different conformations in lipid environments should the need arise.

Without the D-Pro-L-Pro template to serve as the hairpin turn, we chose the Arg-Arg residues in the middle of the sequence and replaced it with a D-Arg-L-Arg sequence since a D-amino acid followed by an L-amino acid has been shown to promote the formation of a hairpin turn. [20] These residues were chosen because they were present at the middle of the sequence and having a turn nucleating sequence in the middle of a loop has been shown to increase the hairpin fold stability of the adjacent residues. [21] The initial variant, acyclin-1, also had three variations within the strands at position 2 (V $\rightarrow$ D), position 11 (L $\rightarrow$ V), and position 13 (L $\rightarrow$ Y). In one instance, the replacement of a Leu, L residue with a Val, V residue at position 11 was studied by Robinson et al. Their results showed that the Val residue increased the AMP activity of mimetic 4 in *E. coli*. Acyclin-1's structural stability was determined using NMR and CD. The Trp/Trp EtF interaction was evident by CD. (Figure S4) The stability of an antiparallel β -hairpin peptide can be quantitated by NMR from the characteristic structuring CSDs of cross-stand H α and H $_N$. (Figure 1) These effects are observed as downfield CSDs for H α 's and H $_N$'s at non-H-bonded and H-bonded sites,

respectively. Acyclin-1 demonstrated the characteristic CSDs of a β -hairpin peptide however the CSD's at these sites were never greater than 0.5ppm indicating that the hairpin fold was not rigidly established. However, the presence of the β -cap interactions can still be verified by observing the CSD of the Gly-H_N. In this case, acyclin-1 had a 1 ppm upfield CSD by NMR which also reflects incomplete folding since a stable β -cap can have up to 3 ppm upfield CSDs at this site. This led to the conclusion that the fold was not yet optimal and could still be improved.

The next variant prepared, acyclin-2, had two β -branched residues replacing 2 cationic residues within the peptide. Since this would have decreased the net positive charge of the peptide, 2 cationic residues were added to the C-terminal end of the peptide to maintain the net positive charge. These variations significantly increased the fold stability of the peptide with downfield shifts of up to 1.2 ppm being observed; the H_N shift comparison of acyclin-1 and -2 clearly shows the greater degree of structuring for acyclin-2 (Figure 1). The CD spectra also demonstrated an increase of intensity at 228 nm going from 87 kdeg·cm²·dmol⁻¹ in acyclin-1 to 134.5 kdeg·cm²·dmol⁻¹ in acyclin-2 (Figure S1, S5). However, despite the improvements in β -strand association, based on the CD and NMR measures, the interactions within the β -cap were still not fully optimized.

In order to further increase the fold stability, a change was made to the hairpin turn. While a D-amino acid followed by an L-amino acid can form a reasonable hairpin turn, the presence of a D-Pro at the turn site should further improve the turn propensity. Acyclin-3 differs from acyclin-2 by this one residue change at the turn. This resulted in a more stable variant, particularly evident as backbone CSDs within the strands as high as 1.5 ppm (Figure S2). The CD melt for acyclin-3 also displayed a larger exciton couplet with a higher melting temperature (Figure 2). These 3 variants were then tested against *E. coli* to determine activity. The results (Table 1, *vide infra*) demonstrate that the AMP activity for acyclin-1 was the highest, with both acyclin-2 and acyclin-3 having higher IC₅₀ values than acyclin-1.

The bioassay results for acyclins 1 – 3 prompted 3 more variants (acyclins -4, -5 and -1p); some key measures of fold population appear in Figure 3. Each of the variants had the same -WpRK-turn as acyclin-3 but had one or both cationic residues within the strands returned in order to identify which of the two were most important for binding and activity. Significant fold stability was attained by our designs; the presence of the more favorable turn was able to rescue the effects of removing a β -branched amino acid in the case of acyclin-4 and -5 (Figure S3). In fact, acyclin-5 showed a 2.5 ppm upfield CSD for the C-terminus Gly-H_N indicating that the β -cap in this peptide was fully formed. However, by CD, the changing of one β -branched residue back to a positive residue resulted in a decrease of the exciton couplet maximum at 228nm for both acyclin-4 and -5. (Figure S6 & S7) Acyclin-1p on the other hand, with both β -branched residues replaced with positive residues, showed an increase in the intensity of the exciton couplet maximum at 228nm, more closely resembling that observed with acyclin-3. (Figure 2 & S8)

We also attempted to improve the fold stability of a second rationally designed AMP, 1-RW. [13] Initial structural measurements of 1-RW showed that the peptide was capable of forming some edge-to-face Trp/Trp interactions. This was evidenced by the rather small

exciton couplet observed by CD. (Figure S11) In addition, 1-RW displayed very few characteristic CSD by NMR indicating that the fold stability was relatively low (Figure S9). In order to improve the stability of the peptide, we replaced the loop, -GSG-, with a hairpin turn, -NRRTGR-. The new turn was chosen because Trp-flanked [4:6] hairpin turns have been shown to be very stable and the residues N, T and G at those specific positions have been shown to be crucial for enhanced hairpin stability. [20] Due to the lack of specific residue preference at the other positions, we chose to use Arg residues in order to increase the net positive charge and thus increase AMP selectivity. This resulted in peptide 2-RW that had a higher fold stability than 1-RW with downfield CSDs of up to 1.5 ppm being observed for backbone H_N (Figure S9). The following variant, 3-RW, included a second stabilizing motif in the form of a β -cap at the termini to decrease terminal fraying. Both 2-RW and 3-RW displayed a much larger CD exciton couplet, see Figure 4 and Figure S12. A graphic comparison of the CD melts appears as Figure S10.

Antibacterial Assay Results

We selected *E. coli* as our representative gram-negative bacterium and *Corynebacterium glutamicum* (*C. glu*) as the gram-positive representative. The difference between the two classes of bacteria lie predominantly in the thickness of the peptidoglycan layer, with gram-positive bacteria having a thick peptidoglycan layer in lieu of an outer membrane. Turning first to the gram-negative data (Table 1), we observed that of the 6 variants that were made for the acyclin series of peptides; the original design, acyclin-1, was the most active AMP with an IC_{50} of 15.85 μ M. The most conserved variant thereof, acyclin-1p, had a higher folded-state population but displayed reduced bioactivity, an approximately 3-fold increase in IC_{50} . This result could be pointing to an important role being played by the Arg residue in the turn either in binding or in providing the necessary overall net positive charge. The importance of the net charge is also highlighted by the lack of activity by acyclins -4 and -5 against *E.coli*. While both peptides have a similar fold stability as acyclin-3, the IC_{50} of both AMPs are extremely high. These peptides both have an even lower net charge than acyclin-1p possibly indicating that the position of the positive residues, be it at the C-terminal tail or within the strands is less important than the overall net charge of the peptide.

This series of peptides was also tested against a gram-positive strain, *C. glu* (Table 2). The results showed that acyclin-4 had the highest activity since the MIC for this peptide was the lowest amongst all 6 derivatives. Acyclin-3 demonstrated similar activity to acyclin-1 at lower concentrations but had a much smaller MIC while acyclin-5 and -1p both had the lowest activity. When taken as a whole, peptides that had an Arg residue at position 10 seem to be less effective against *C. glu* than peptides with a Val residue at that position. This could be an indication that in gram-positive systems, the overall net charge is less important since acyclin-4 has a lower overall net charge than acyclins -3 and -2 and yet has the highest activity.

Based on these results, it would seem that, in this series, there are quite different requirements for activity optimization against our gram-positive and gram-negative test species. The results would seem to indicate that the N-terminus Arg residue that was replaced plays an important role in the activity of the peptide against gram-positive cell

walls since acyclin-4 that has an Arg at position 3 is slightly more potent than acyclin-3 which has a Val at this position. It is also possible that the higher level of structuring of the C-terminal strand of these peptides plays a role in the activity indicating a possible relationship between C-terminal β -sheet propensity and activity against gram-positive cell walls

As for the RW series, the improved hairpin structure of 2-RW resulted in a slight improvement in AMP activity in *E. coli*. The IC_{50} of 2-RW was lower than that of 1-RW. However, the addition of the more hydrophobic β -cap, while improving structure at the termini, dramatically reduced the activity of 3-RW against *E. coli*. The removal of the N-terminal cationic residue may indicate an important role being played by this position. The likelihood that the net positive charge is the contributing factor is low since 2-RW has a similar level of activity as 1-RW despite having a higher net positive charge.

While the gram-negative data showed a lack of improvement in activity for the variants, the gram-positive data shows that the variant 3-RW is more potent than the native peptide. The MIC of 3-RW was slightly lower than that of 1-RW but the differences between the activity of the two peptides becomes much more obvious at the lower peptide concentrations (0.05 – 0.2 μ M), see Figure 5. Unfortunately, due to solubility issues, we were unable to ascertain the activity of 2-RW against our control gram-positive bacteria, *C. glu*.

Conclusion

Through this study, we have determined that non-covalent stabilizations can improve the fold of β -hairpin AMPs that previously depended on covalent stabilizations from disulfide cross-links to maintain secondary structure and bioactivity. While we were able to synthesize a series of variants that were stable hairpins, there was no reliable correlation between hairpin stability and biological activity against *E. coli*. Our initial variant design, acyclin-1, appears to have the highest activity and subsequent hairpin-stability improvements resulted in decreased AMP activity. Even with minor changes, antibacterial activity was almost entirely abolished, as in the case of acyclin-4. The bioassay data for the RW series of peptides reinforce the lack of a direct correlation between hairpin structure stability and activity. While 2-RW and 3-RW demonstrated very similar hairpin fold stability by NMR, the activity of these two peptides was vastly different against *E. coli* indicating that structure alone is not a good predictor of activity. It is also worth noting that despite the much more structured nature of 2-RW, the IC_{50} of this peptide against *E. coli* is not much lower than the less structured 1-RW starting point.

However, we were able to significantly increase the potency of the AMPs against a gram-positive system, *C. glu*. We were also able to clarify a possibly important role being played by position 10 of the peptide since all three variants with a Val residue at this position demonstrate the highest level of activity against the gram-positive cell wall.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.
Histograms depicting the chemical shift deviation, CSDs, of the backbone H_N sites of for acyclin-1 and acyclin-2 at each residue at 280K.

Figure 2.
CD spectra of acyclin-3 taken every 10°C between 5°C and 95°C.

Figure 3.

Histograms depicting the backbone H_N CSDs of acyclin-4 and acyclin-1p at 320K. The lack of a β -branched residue at position 10 results in a dramatic decrease in folded mole fraction for acyclin-1p at 320K.

Figure 4. The CD spectra of 2-RW taken between 5°C to 95°C. Measurements were taken at 10°C intervals.

Figure 5.

The % growth (versus a non-inhibited control) of the gram-positive bacterium, *C. glu.* peptides as modified by different concentrations of 1-RW and 3-RW. The MIC represents the concentration of peptide that completely abolishes bacterial growth (% growth $\cong$ 0). The superiority of 3-RW throughout the 0.05 – 0.3 μ M range is evident.

Table 1.

Sequences of the peptides that were synthesized and their melting temperature, T_M (as calculated by CD at 228 nm) as well as the chemical shift deviation, CSD of the C-terminal Gly- H_N that is part of the β -cap (as observed by NMR at 280K). The residues in bold depict the variations made to each relative to acyclin-1 and 1-RW. The presumptive turn residues in the central portion of the sequence are set off by hyphens. N/A = not applicable

Peptide	Sequence	T_M (°C)	CSD (ppm)
Acyclin 1	Bz – WIRYK– WrRK –KVERYWTGP –NH ₂	< 0	–0.925
Acyclin 1p	Bz – WIRYK– W p RK –KVERYWTGP –NH ₂	35.5	–0.651 **
Acyclin 2	Bz – WIVYK– WrRK – V VERYWTG KR –NH ₂	55.1	–0.375
Acyclin 3	Bz – WIVYK– W p RK – V VERYWTG KR –NH ₂	>100	
Acyclin 4	Bz – WIRYK– W p RK – V VERYWTGP –NH ₂	64.5	–1.162
Acyclin 5	Bz – WIVYK– W p RK –KVERYWTGP –NH ₂	73.9	–2.450
1-RW	Ac–RWTW–RGS–GR–WTWR–NH ₂	78.9	N/A
2-RW	…RWRW– NRRT GR– WRWY	27.8	N/A
3-RW	Bz– WRW – NRRT GR– WRW TGP–NH ₂	31.7	–2.735

** The CSD for acyclin-1p Gly- H_N is given for 320K. This is due to the fact that NMR taken at lower temperature displayed large amounts of peak broadening that resulted in an inability to record CSDs for the sample at lower temperatures.

Table 2.

The minimum inhibitory concentration range of the peptides against the gram-positive bacteria, *C. glu* and gram-negative bacteria, *E. coli*. The IC₅₀ values against both bacteria is also given. n.t. = not tested

Peptide	<i>C. glu</i>		<i>E. coli</i>	
	MIC (μM)	IC ₅₀ (μM)	MIC (μM)	IC ₅₀ (μM)
Acyclin 1	0.9 – 0.7	0.06	25	16
Acyclin 1p	0.9 – 0.7	0.1	> 100	45
Acyclin 2	0.1 – 0.05	0.02	>100	40
Acyclin 3	0.2 – 0.1	0.04	100	44
Acyclin 4	0.05 – 0.02	0.01	>100	>100
Acyclin 5	0.9 – 0.7	0.13	>100	95
1-RW	0.9 – 0.7	0.05	25	12
2-RW	n.t.	n.t.	12.5	8
3-RW	0.5 – 0.4	0.02	50	39