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Bioassays of Analogs of Pleurocidin-Amide Indicate that Change at *N*-Terminus is Key to Improved Efficacy

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Introduction

We have a problem. Many microbial pathogens are becoming resistant to conventional therapeutics, and are no longer affected by them. We need to develop the next generation of antimicrobials or return to times when a minor bacterial infection could be fatal. Antimicrobial peptides (AMPs) typically have broad-spectrum specificity, which makes them ideal for critical treatment situations when the identity of the pathogen is unknown. Pleurocidin, an AMP found in secretions from winter flounder (*Pleuronectes americanus*), adopts an amphipathic α -helical structure crucial for activity, and this conformation is induced or stabilized by peptide-membrane interactions [1]. We evaluated the impacts of novel pleurocidin analogs on the exponential growth curves of two Gram (+) and two Gram (-) human pathogens using high-throughput broth bioassays capable of simultaneous monitoring the growth of multiple bacterial species on a single 96-well plate [2]. Our hypothesis holds that specific additions to the *N*-terminus of our “base peptide” pleurocidin-amide, (LM4-12), which were designed to enhance initiation of peptide-cell membrane interactions, will increase antimicrobial efficacy and selectivity [3]. We have tested the effects of grafting combinations of **R**, **G**, & **W** onto the *N*-terminus of base peptide LM4-12, synthesized using Boc-SPPS methods on an MBHA resin [4]. Comparison of the effects of adding each of these three amino acids to the *N*-terminus of LM4-12 generated the lead peptide LM4-15, with an *N*-terminal arginine, that yielded dramatically improved broad-spectrum efficacy against *Enterococcus faecalis* (EF), *Staphylococcus aureus* (SA), *Pseudomonas aeruginosa* (PA) and *Escherichia coli* (EC).

LM4-15 (*N*-Ter) H-RGWGSFFKKAHVGVGKAALTHYL-NH₂ (*C*-Ter)

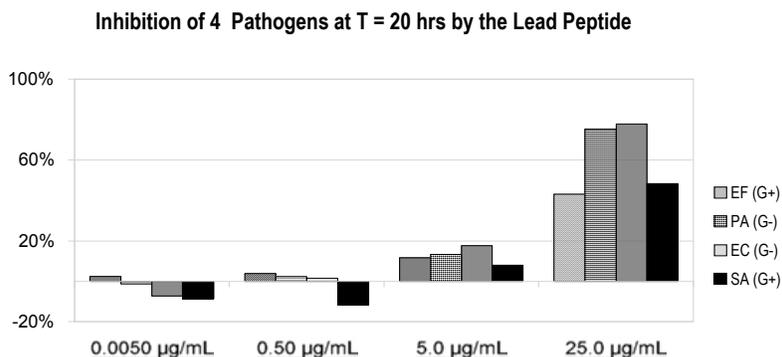


Fig. 1. Lead Peptide LM4-15.

Results and Discussion

Lead peptide LM4-15 (Figure 1) showed a significant efficacy against all four human pathogens, with $\geq 48\%$ growth inhibition relative to untreated bacterial cultures at the maximum tested dose (25 $\mu\text{g/mL}$, 8.72 μM). Base peptide LM4-12 (Figure 2) showed a similar inhibitory effect, but only on two of the four pathogens (both Gram +), and some species selectivity, with the highest efficacy against SA. Growth inhibition was calculated as the OD₆₃₀ of peptide-treated bacteria, divided by the OD₆₃₀ of identical untreated cells grown in broth cultures, under the same conditions, measured 20 hours after a single treatment with the test peptide at the indicated dose.

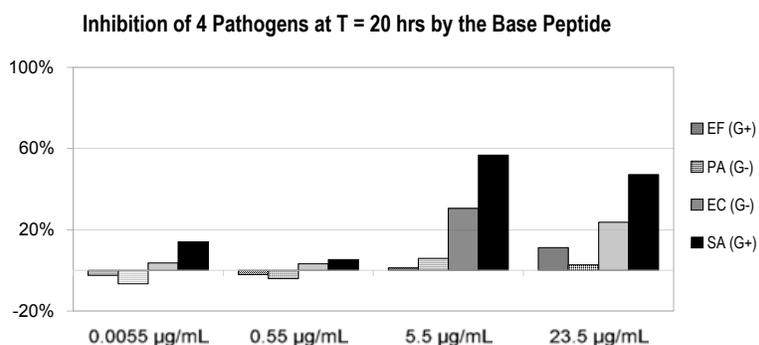


Fig. 2. Base Peptide **LM4-12**.

MIC assays typically test the effectiveness of potential antibiotics using a fairly low (10^4 CFU) inoculum of bacteria. Our data, obtained at a higher starting concentration of bacteria ($OD_{630} = 0.12$ at $t = 0$), shows that **LM4-12** reached a maximum efficacy for SA, and EC at the second highest dose of the base peptide tested ($5.5 \mu\text{g/mL}$, $1.85 \mu\text{M}$), but was not effective against either PA or EF, even at the highest tested dose ($23.5 \mu\text{g/mL}$, $8.64 \mu\text{M}$). **LM4-15** however, was found to reach a much higher efficacy against all 4 target species at the maximum tested dose of the lead peptide ($25.0 \mu\text{g/mL}$, $8.72 \mu\text{M}$), attaining an efficacy of greater than 48% growth inhibition when tested on gram positive microorganisms SA and EF. What is more, **LM4-15**, at the highest tested dose, showed greater than 73% growth inhibition when tested on the gram negatives, EC and PA, which were not inhibited by **LM4-12** to any great extent (Figures 3-6).

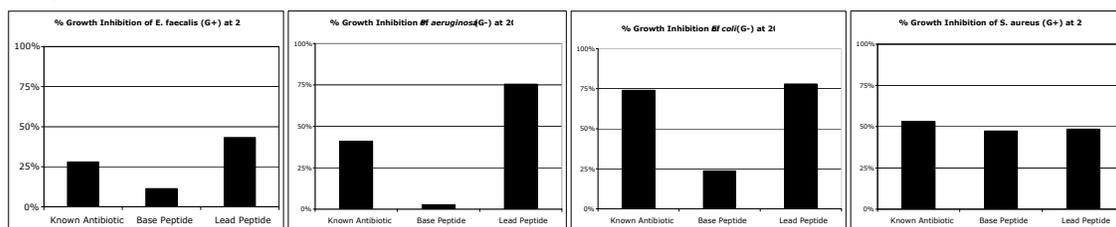


Fig. 3. *EF* (G+).

Fig. 4. *PA* (G-).

Fig. 5. *EC* (G-).

Fig. 6. *SA* (G+).

In conclusion, modifying the *N*-terminus of the base antimicrobial peptide by adding arginine dramatically enhanced antimicrobial efficacy in the lead peptide **LM4-15** over that of the base peptide **LM4-12**. As the assay data in Figures 1 and 2 demonstrates, peptide doses as low as $5 \mu\text{g/mL}$ slightly retard bacterial growth, with a significant level of inhibition reached at only $25 \mu\text{g/mL}$, the maximum dose assayed in these experiments. Comparing the efficacies of the lead and base peptides with those of a known antibiotic, oligomycin, confirmed that the lead peptide inhibited bacterial growth of three of the four pathogenic microbial species tested (*EF*, *PA*, and *EC*) as well as (or better than) the known antibiotic in our assays. This lead peptide shows great promise as a therapeutic agent, especially now that more bacteria are becoming increasingly resistant to conventional antibiotics. The dramatic increase in antibiotic efficacy observed with a single arginine addition onto the *N*-terminus of pleurocidin in **LM4-15**, versus the low efficacy of similar synthetic peptide analogs prepared with tryptophan, lysine, and glycine at that position, suggests that *N*-terminal arginine addition onto existing AMPs may prove to be a general strategy for increasing AMP effectiveness in targeting diverse bacterial membranes.

References

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