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TITLE: Evaluation of Novel Antimicrobial Peptides as Topical Anti-Infectives with Broad-Spectrum Activity against Combat-Related Bacterial and Fungal Wound Infections

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14. ABSTRACT

Ballistic wound infection has become the greatest threat to the life and recovery of the combat casualty who survives the immediate trauma of the insult. Multidrug resistance and generation of recalcitrant biofilm are major obstacles in treating wounds. Antimicrobial peptides (AMPs), also known as host defense peptides, are evolutionarily highly conserved components of the innate immune system that provide the first line of defense against invading pathogens in all multicellular organisms. Designed antimicrobial peptides (dAMPs) are synthesized peptides that have been rationally designed based on sequences found in naturally occurring AMPs. dAMPs are amphipathic cationic peptides with the ability to kill microbes by disrupting their membrane function. This mode of action rapidly kills antibiotic resistant microbes, even in biofilm. Bacteria are thus less likely to develop resistance to AMPs.

Riptide Bioscience has synthesized thirty-one novel dAMPs in four iterative rounds. The dAMPs were evaluated for their antimicrobial potency against 11 strains of gram positive and gram negative bacteria and 7 strains of fungi. Standard MIC (Minimum Inhibitory Concentration) assays were conducted according to CLSI guidelines. Cytotoxicity was determined using L929 fibroblasts into which the luciferase gene had been transfected. Time-kill assays used *P. aeruginosa* (Xen5) and *S. aureus* (Xen36) cultures.

- dAMP sequences have been developed that have bactericidal and anti-fungal activity
- Rapidly acting antimicrobial peptides have been developed that when applied topically to infected wounds, have the potential to eradicate infection, preserve tissue, enhance healing and reduce the opportunities for systemic infection.
- Three dAMPs have been selected for evaluation in a porcine burn wound model infected with *P.aeruginosa* and *S. aureus*.

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1. Introduction

The relentless growth of multidrug resistance bacteria and generation of recalcitrant biofilm are major obstacles in treating wounds. Modern combat wounds are particularly troublesome, compared to peacetime traumatic injuries because the higher velocity projectiles inflicted by IEDs causes more severe injury and accompanying wounds, including burns, are frequently contaminated by pathogenic bacteria and fungi.

Burn wounds, in the absence of topical antibiotics, are immediately colonized by gram-positive skin flora, such as *Staphylococcus aureus*. Gram-negative bacteria such as *Pseudomonas aeruginsoa*, *Klebsiella pneumoniae*, and *Escherichia coli*, from the patients' respiratory and gastrointestinal tract, typically colonize the wound 48 to 72 hours post injury. *S. aureus* and *P. aeruginosa* are the culprit pathogens which are most likely to result in an invasive infection shortly after burn injury. Multidrug resistance is common thereby limiting antibiotic therapy options.

Wound infection prevention in the form of topical antibiotics and early debridement has been associated with a large reduction in burn wound infections. Current topical antibiotics include mafenide acetate, silver sulfadiazine or silver nitrate, and silver-impregnated dressings, however, these agents have limitations and inherent risks of complications. Silver sulfadiazine is not active against fungal infections, and its side effects include staining of the treated burn wound, allergic reactions to the sulfadiazine moiety and delays in the rate of burn wound healing. Similar to silver sulfadiazine, silver nitrate solution penetrates poorly into eschar, requires the use of occlusive dressings, and turns black upon contact with tissues. Mafenide acetate causes pain upon application, is not effective against fungal infections, and it and its main metabolite are inhibitors of carbonic anhydrase and have been known to cause metabolic acidosis. Although traditional topical antimicrobial agents have had some success in treating wounds, given the increased occurrence of multidrug resistance and inactivity against fungal infections innovative developments are desperately needed.

To meet the challenge of treating infected wounds with topical antimicrobial and anti-fungal agents Riptide Bioscience is developing designed antimicrobial peptides (dAMPs). Designed antimicrobial peptides (dAMPs) are synthesized peptides that have been rationally designed based on sequences found in naturally occurring AMPs. dAMPs are amphipathic cationic peptides with the ability to kill microbes by disrupting their membrane function. This mode of action rapidly kills antibiotic resistant microbes, even in biofilm. Bacteria have never succeeded in developing resistance to a variety of AMPs.

2. Keywords

Antimicrobial, peptides, anti-fungal, wounds, burns, bacterial resistance, antibiotics, AMP, biofilm, infection, amphipathic, dAMP.

3. Accomplishments

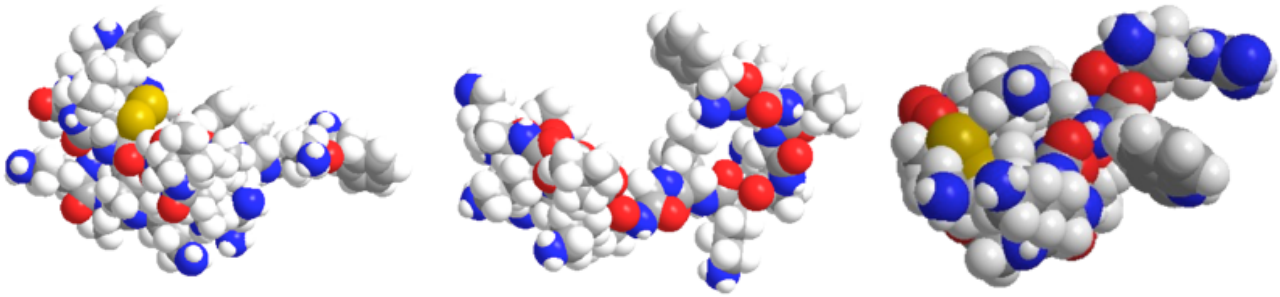
Riptide Bioscience has synthesized thirty-one novel dAMPs in the four iterative rounds. The dAMPs were evaluated for their antimicrobial potency against 11 strains of bacteria and 7 strains

of fungi. Standard MIC (Minimum Inhibitory Concentration) assays were conducted according to CLSI guidelines. Cytotoxicity was determined using L929 fibroblasts into which the luciferase gene had been transfected. Time-kill assays used *P. aeruginosa* (Xen5) and *S. aureus* (Xen36) cultures. Both the cytotoxicity and time-kill assays were developed and used for the first time to measure those parameters for this study. The use of bioluminescence as measured by IVIS provides reproducible real-time data on cell viability. As a result, from the assays conducted to date the following has been accomplished:

- dAMP sequences have been developed that have bactericidal and anti-fungal activity
- Rapidly acting antimicrobial peptides have been developed that when applied topically to infected wounds, have the potential to eradicate infection, preserve tissue, enhance healing and reduce the opportunities for systemic infection.
- Three dAMPs have been selected for evaluation in a porcine burn wound model infected with P.aeruginosa and S. aureus.

The three peptides selected to be evaluated for their effectiveness in an infected porcine burn wound model are RP504, JC41-4 and TY13. RP504 is an amphipathic peptide with 23 amino acids of which seven are ornithine. This peptide also has one cysteine-cysteine bond that results in the formation of a hairpin loop. JC41-4 is an amphipathic peptide with 17 amino acids of which eight are ornithine residues. It has an alpha-helical formation. TY13 is also an amphipathic peptide with 17 amino acids. It is a tachyplesin-like peptide with two sets of cysteine-cysteine bonds. It has a tight hairpin shape.

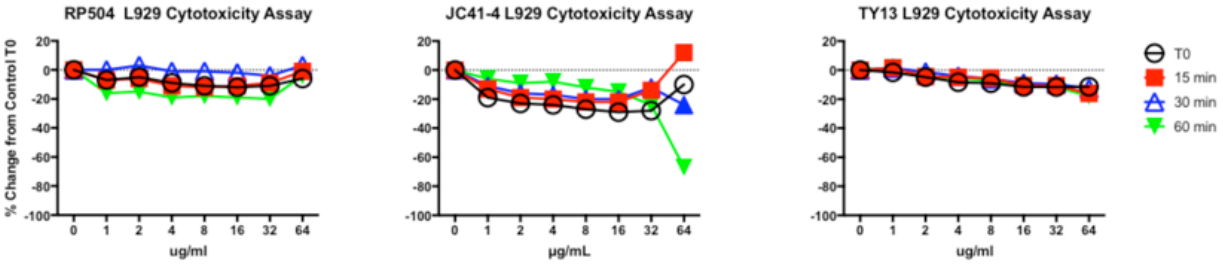
RP504 **JC41-4** **TY13**



Limited Cytotoxicity to Mammalian Cells

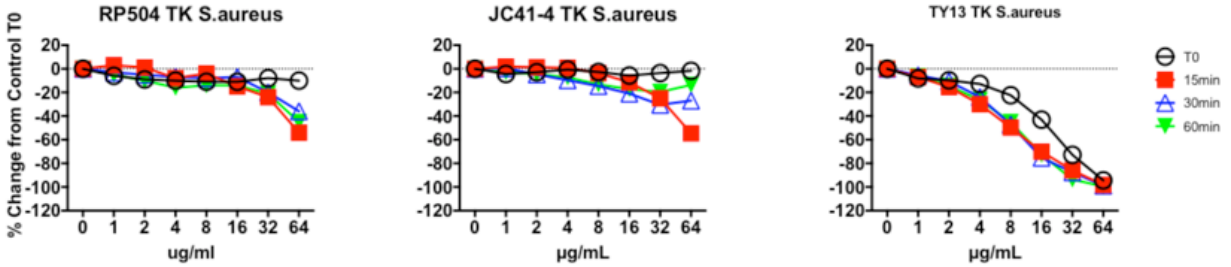
Mammalian cytotoxicity was determined using muring fibroblast L929 cells expressing the bioluminescent reporter, luciferase (luc) gene. L929-luc cells plated in 96 well plates at $1x10^4$ cells/well, incubated overnight with test compounds added. Bioluminescence measured with the IVIS Lumina system (Perkin Elmer). Data represents the mean of three replicates and is expressed as the % change from T0.

RP504 JC41-4 TY13



dHDPs Rapidly kill S.aureus

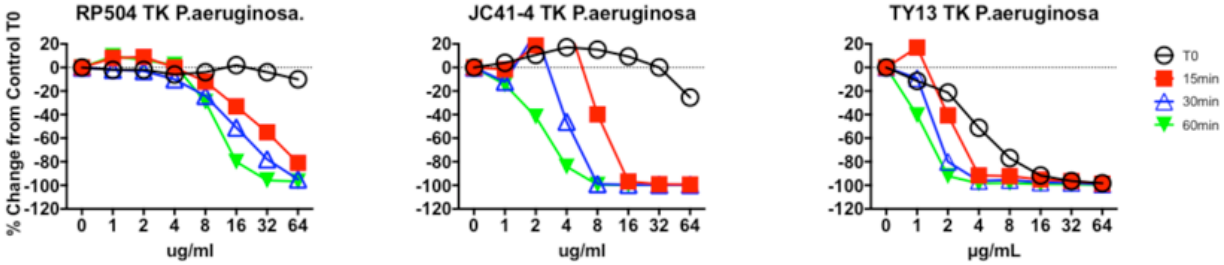
RP504 JC41-4 TY13



Time-kill assay using bioluminescent *S.aureus* pathogenic bacteria (Xen36, Perkin Elmer). Data represent the mean of three measurements.

dHDPs Rapidly Kill P.aeruginosa

RP504 JC41-4 TY13



Time-kill assay using bioluminescent *P. aeruginosa* pathogenic bacteria (Xen5, Perkin Elmer). Data represent the mean of three measurements.

The rapid killing of bacteria, as demonstrated in the time-kill assays, suggests that the dHDPs kill by disrupting membrane function. This is consistent with the amphipathic properties of the dHDPs and is a mechanism to which resistance is difficult to develop.

The time-kill assays were performed as a screening test on only two strains of bacteria as a way of identifying peptides that were further evaluated in MIC assays against a range of gram positive and gram negative bacteria and fungal strains.

G- Bacterial Strain MIC Values (µg/mL)

Bacteria	RP504	JC41-4	TY13	Tobramycin	Vancomycin
A. baumannii (G-)					
6043	32	8	8	64	32
6838	64	4	8	2	128
ATCC 17978	32	4	16	2	32
E. cloacae (G-)					
6053	32	4	8	32	64
6054	32	2	8	128	64
K. pneumonia (G-)					
6066	128	128	8	2	128
6069	32	16	4	2	128
ATCC 10031	32	8	8	2	64
P. aeruginosa (G-)					
6186	16	8	4	128	128
ATCC 19660	16	8	4	2	128
ATCC 27853	16	4	4	2	128

Observations: TY13 has a broader and more effective range of growth inhibiting activity of G- bacterial strains than RP504 or JC41-4. Each of the peptides is more effective over the range of G- strains tested than isVancomycin and in several cases is more effective than Tobramycin.

G+ Bacterial Strain MIC Values (µg/mL)

Bacteria	RP504	JC41-4	TY13	Tobramycin	Vancomycin
S. aureus (G+)					
B-767	32	4	4	128	64
6061	64	32	8	2	2
MRSA (G+)					
6313	64	32	4	128	2
6381	64	32	4	2	2
ATCC 33592	32	32	4	2	2
S. epidermis (G+)					
ATCC 51625	16	2	8	2	2

Observations: TY13 has lower MIC values against G+ bacterial strains than RP504 or JC41-4

Fungal Strain MIC Values (µg/mL)

Fungi	RP504	JC41-4	TY13	Voriconazole	Amphotericin
Candida albicans					
Y-326	8	32	16	10	1
Y-6359	4	32	4	30	1
C. parapsilosis					
Y-1761	8	16	16	0.3	1
Y-1763	2	8	2	1	1
Candida krusei					
Y-27803	8	16	16	1	1
Y-27825	8	16	16	1	1
A. fumigatus					
9648	128	64	64	1	10
9651	128	32	64	0.3	30
A. flavus					
MYA-3651	128	32	64	0.3	10
MYA-1004	128	64	64	0.3	10
A. carymbifera					
NRRL 6251	8	4	2	10	3
F.solani					
NRRL 28548	2	2	2	10	10
M. circinelloides					
NRRL 3631	128	32	64	30	1

Observations: While RP504 has the lowest MIC values among the three dHDPs against the six strains of Candida, JC41-4 has the overall broadest range of effectiveness. However each peptide has a broad range of effectiveness against the fungal strains evaluated in this *in vitro* assay.

Conclusions: The rapid dose-response demonstrated in the time-kill studies suggests that the dHDPs are killing bacteria by disrupting essential membrane functions. It is highly unlikely that bacteria will develop resistance to this mechanism of bactericidal activity. Moreover, the minimal cytotoxicity demonstrated against mammalian cells demonstrates a selective preference for prokaryotic cell membranes. With their preference for selectively disrupting microbial membrane functions, it is expected that the peptides will have a therapeutic index that will enable them to effectively eliminate infective agents and promote wound healing. From the MIC assays three dHDPs, each with broad a broad range of effectiveness have been selected to be evaluated in a porcine burn wound model.

4. Impact

The identification of three very different structures that are effective against a wide range of bacteria and fungi is encouraging. If wounds infected with multiple microbes can be treated effectively with one agent rather than agents that are specific for each strain infecting a wound, there is less chance for systemic infection and the opportunity for more rapid healing. By disrupting microbial membrane function the chance of microbes developing resistance is low.

5. Changes/Problems

Trideum Bioscience was originally contracted to perform the *in vivo* evaluation of selected dHDPs in a mouse burn wound model. However, during this past year we were able to determine that the *in vivo* evaluation could be done in a porcine burn wound model without any change in the overall budget. The porcine wound model is more similar to human wounds than a rodent model and thus we have obtained permission to proceed with studies at Bridge PTS in Austin, TX.

6. Products

Poster #1054 presentation at MHSRS 2016 First Prize Award

7. Participants and other Collaborating Organizations

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8. Special Reporting Requirements

None

9. Appendix

None