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PRINCIPAL INVESTIGATOR: Louis Edward Clemens

CONTRACTING ORGANIZATION: Riptide Bioscience, Inc.
Vallejo, CA 94592

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Louis Edward Clemens

E-Mail: eclemens@riptidebio.com

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Riptide Bioscience, Inc.
941 Railroad Ave. Vallejo, CA 94592

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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 have never succeeded in developing resistance to a variety of AMPs.

Riptide Bioscience has synthesized six novel dAMPs in the first of three iterative rounds of planned studies. 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.

- dAMP sequences have been developed that have bactericidal and anti-fungal activity
- Three dAMPs have been shown to quickly kill bacteria without causing fibroblast cytotoxicity
- 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

15. SUBJECT TERMS dAMP,AMP bactericidal, anti-fungal, wounds, infection, biofilm, bacterial resistance, fungi,					
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Riptide Bioscience Annual Report 2016

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,[4] 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.

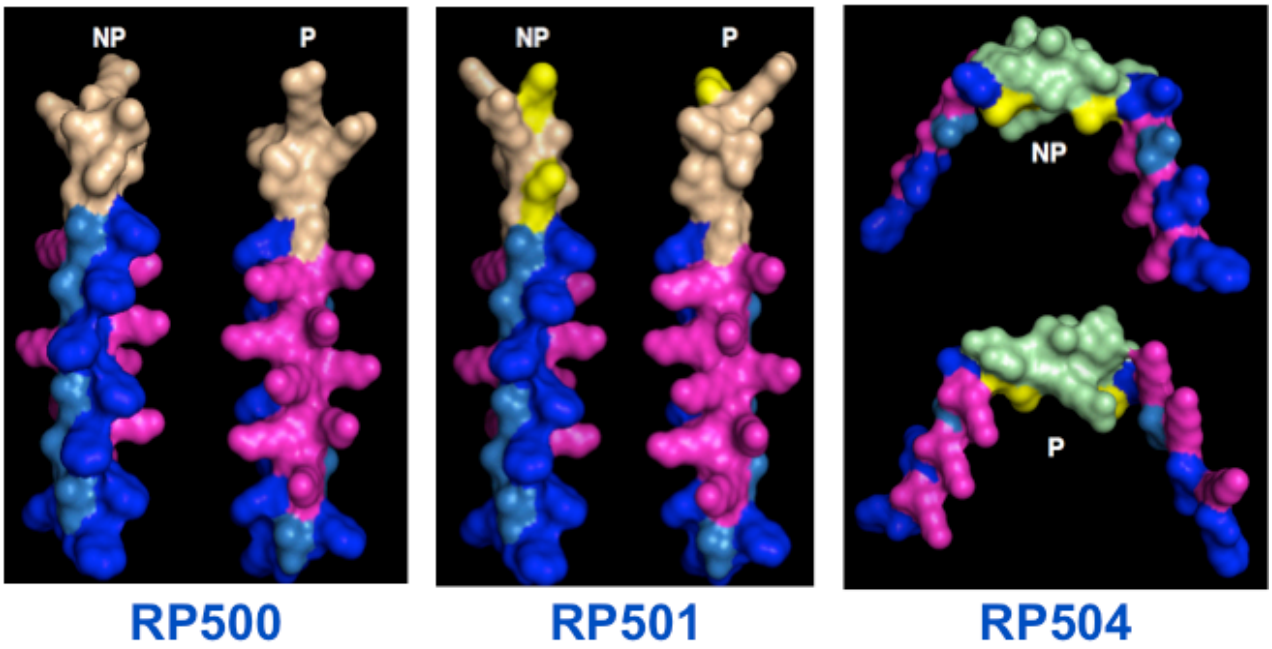
3. Accomplishments

Riptide Bioscience has synthesized six novel dAMPs in the first of three iterative rounds of planned studies. 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:

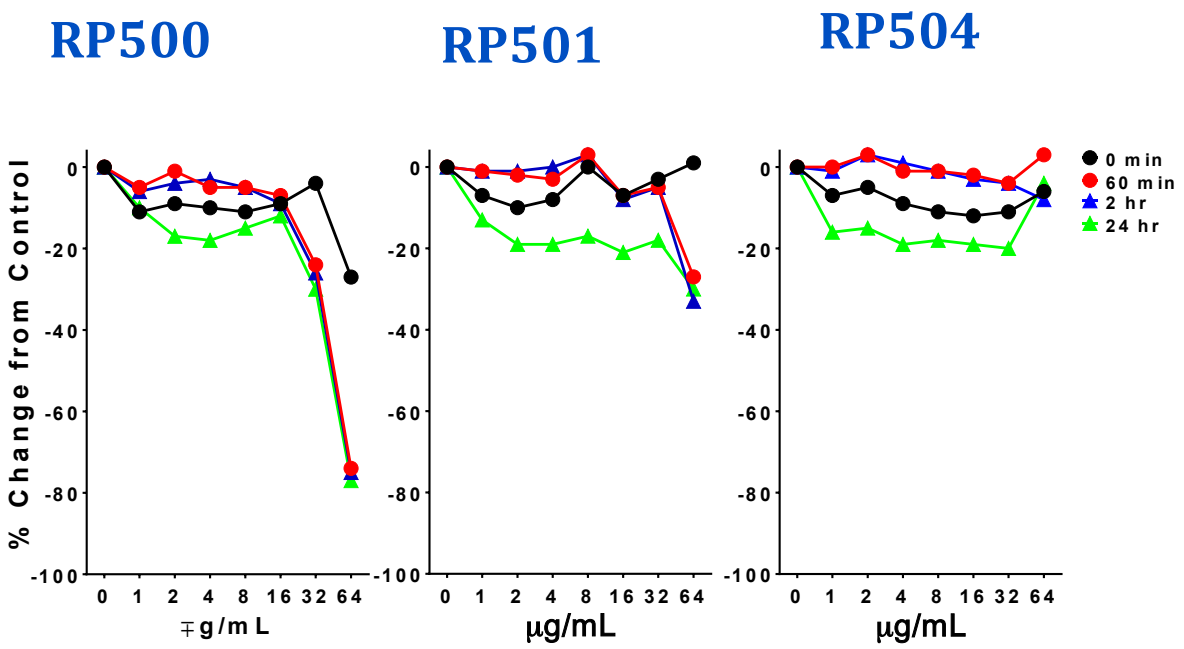
- dAMP sequences have been developed that have bactericidal and anti-fungal activity
- Three dAMPs have been shown to quickly kill bacteria without causing fibroblast cytotoxicity
- Rapidly acting antimicrobial peptides have been developed that when applied topically to infected wounds, have the potential to eradicate infection, preserve tissue, enhance healing and reduce the opportunities for systemic infection.

The three most active peptides assayed in the first of three design and synthesis iterations are:



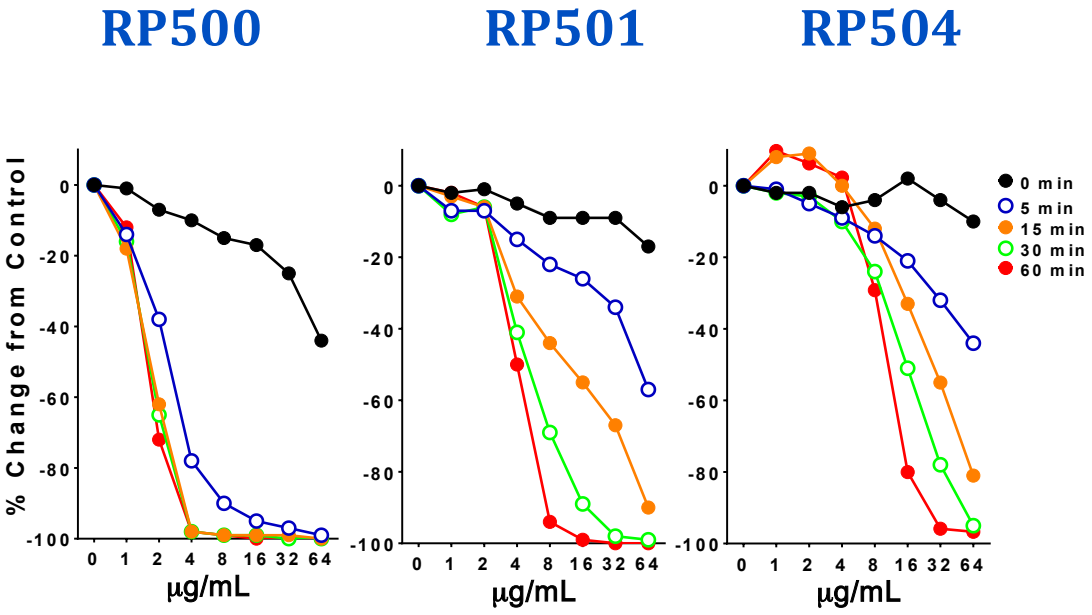
The AMPs are amphipathic, α-helical molecules, having a NP (**non-polar hydrophobic face**) and a P (**polar hydrophilic face**). RP500 and RP501 are linked to a LPS binding sequence by two glycine amino acids. The positive charges on the polar surface are attracted to the negatively charged phospholipids on the cell membranes of microorganisms. RP504 is a Tachyplesin-like design. Tachyplesin is an AMP found in a Japanese horseshoe crab.

Limited Cytotoxicity to Mammalian Cells



Mammalian cytotoxicity determined using murine fibroblast L929 cells expressing the bioluminescent reporter, luciferase (luc). L929-luc cells plated in 96 well plates at $1x10^4$ cells/well, incubated overnight & test compounds added. Bioluminescence measured with the IVIS Lumina system (Perkin Elmer). Data represent the mean of three measurements

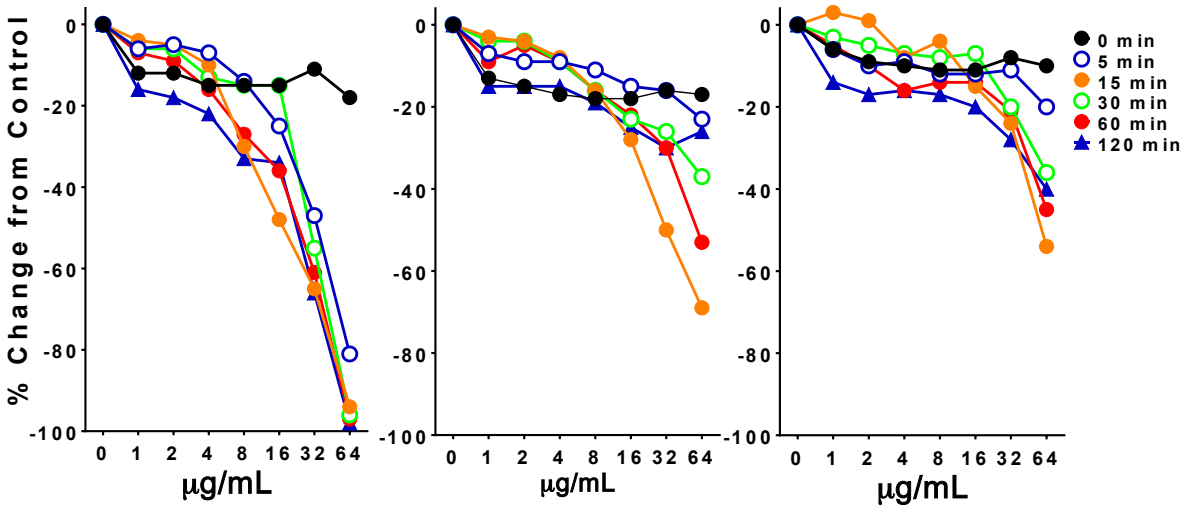
AMPs Rapid Kill *P. aeruginosa*



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

AMPs Rapidly Kill *S. aureus*

RP500 RP501 RP504



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

Bacterial Strain MIC Values (µg/mL)

Bacteria	RP500	RP501	RP504	Tobramycin	Vancomycin
***A. baumannii* (G-)**					
6043	16	32	32	16	>128
6838	64	32	64	2	>128
ATCC 17978	32	16	32	2	>128
***E. cloacae* (G-)**					
6053	32	32	32	32	>128
6054	32	64	32	64	>128
***K. pneumonia* (G-)**					
6069	32	32	32	2	>128
ATCC 10031	32	32	32	2	>128
***P. aeruginosa* (G-)**					
6186	32	32	16	>128	>128
ATCC 19660 Wild type	32	64	16	>128	>128
ATCC 27853 Cytotoxic	32	64	16	2	>128
***S. aureus* (G+)**					
B-767	16	32	32	>128	>128
6061	32	64	64	4	2
MRSA (G+)					
6313	32	64	64	>128	2
6381	32	32	64	128	2
ATCC 33592	32	32	32	64	2
***S. epidermis* (G+)**					
ATCC 51625	16	16	16	2	2

Fungal Strain MIC Values (µg/mL)

Fungi	RP500	RP501	RP504	Voriconazole	Amphotericin
Candida albicans					
Y-326	>128	>128	8	0.1	0.3
Y-6359	>128	>128	4	0.1	0.3
Candida parapsilosis					
Y-1761	64	128	8	0.3	1
Y-1763	>128	>128	2	0.3	1
Candida krusei					
Y-27803	32	32	8	0.3	1
Y-27825	32	32	8	0.3	1
Absimia carymbifera					
NRRL 6251	8	8	8	30	3
Fusarium solani					
NRRL 28548	4	16	2	10	30

Conclusions: The rapid dose-response demonstrated in the time-kill studies suggests that the dAMPs are killing bacteria by disrupting essential membrane functions. It is highly unlikely that bacteria will develop resistance to this mechanism of bactericidal activity. Of the peptides tested in this iteration, the tachyplesin-line peptide, RP504 has the broadest range of antimicrobial activity. It is also the least cytotoxic peptide. This is a structure that will serve as a model for designing peptides in the next iteration.

4. Impact

The identification of a structure that is 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. The time-kill data suggests that in a wound, as the dAMP is metabolized and/or diluted with tissue fluids, it will continue to be active. We are aware that the peptides will be degraded by lytic enzymes and will modify their structure to be more resistant to proteolysis.

5. Changes/Problems

During this first set of assays it took some time to set up and validate the MIC assays, and to develop the L929 cell line used in the cytotoxicity assay with the luciferase gene. We've now established these assays and have also standardized the time-kill assays with the luminescent bacterial strains. We have also changed vendors for the synthesis of our peptides in order to achieve faster and cheaper service.

6. Products

Poster #1054 presentation at MHSRS 2016 First Prize Award

7. Participants and other Collaborating Organizations

Dr. L. Edward Clemens – PI and PO
eclemens@riptidebio.com
415-710-6282

Dr. Kathryn Woodburn – Consultant – Data Analysis
Person Months - 2
kwoodburn@riptidebio.com
408-805-2982

Dr. Jesse Jaynes – Consultant – Chemist
Person Months 1.5
jjsqrd@bellsouth.net
334-332-1527

Trideum Bioscience – Contractor –Performs MIC Assays
Mina Izadjoo
mizadjoo@trideum.com
301-204-4201

Lumigenics LLC – Contractor – Performs Cytotoxicity and Time-Kill Assays
Ed Lim
Ed.lim@lumigenics.com
925-338-4411

8. Special Reporting Requirements

None

9. Appendix

None