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

- Riptide Bioscience has developed a unique Time-Kill assay using bioluminescent bacteria. This assay demonstrates the time needed to eliminate microbes *in vitro* as well as insight into the bactericidal mechanism of action of test compounds.
- In vitro MIC and MBC assays have demonstrated that S. aureus, P. aeruginosa, and several strains of fungi do not develop resistance after repeated passage with dAMPs, whereas strains resistant to the antibiotics gentamicin, clindamycin or fluconazole are killed by dAMPs. The dAMPs also kill bacteria and fungi in their biofilm.
- Three dAMPs (RP504, RP554 and RP557) were evaluated in a porcine burn wound model for their ability to stop polymicrobial infections. RP557 was the most effective peptide.
- Three dAMPs were evaluated for antifungal activity in a rat model of vulvovaginal candidiasis. R557 was the most effective in eradicating the infection.
- RP557 has very limited cytotoxic effects on eukaryotic cells.
- RP557 is being advanced to IND enabling studies.

15. SUBJECT TERMS dAMP,AMP, bacteria, fungi, MIC, Time-Kill, Burns, Infection, Porcine, bacterial resistance, amphipathic, peptide					
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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 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
- Several dAMP sequences have the ability to eradicate bacteria and fungi in their preformed and mature biofilm.

- 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 RP504, RP554 and RP557 have been evaluated in a porcine burn wound model infected with both P.aeruginosa and S. aureus.
- Three dAMPs were evaluated in a vulvovaginal *in vivo* model of fungal infection.
- RP557 has been selected for IND enabling studies.

The three peptides selected for evaluation in an infected porcine burn wound model and in the rat vulvovaginal model of a fungal infection were RP504, RP554 and RP557 **(Figure 1**). 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. RP554 is an amphipathic peptide with 17 amino acids of which eight are ornithine residues. It has an alpha-helical formation. RP557 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.

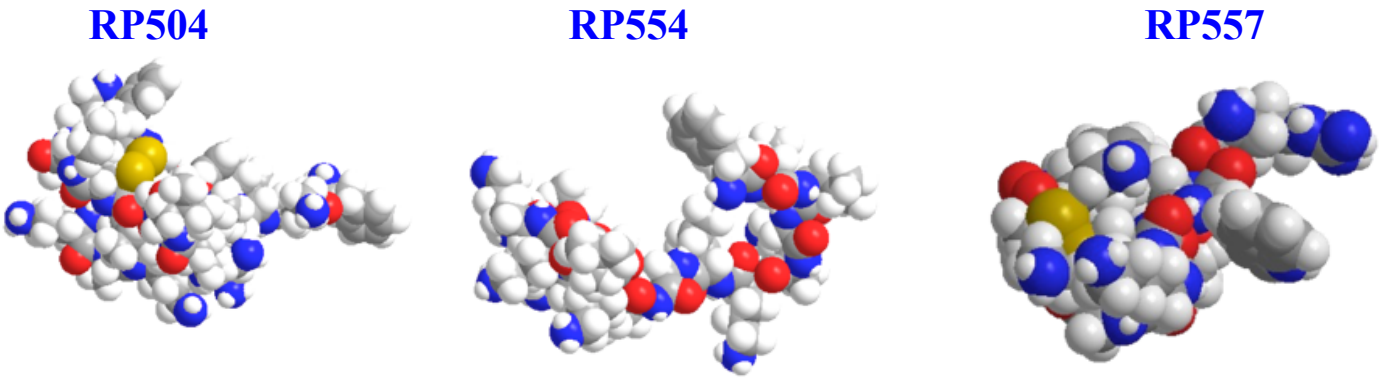


Figure 1. Three dAMPs selected for evaluation in a polymicrobial bacterial infected porcine burn wound model and in the vulvovaginal study of an in vivo fungal infection.

RP557 has been selected for IND enabling studies because it has demonstrated effectiveness in killing a wide range of antibiotic resistant bacteria and fungi. It is a bactericidal agent that has a high degree of specificity for microbes and a low level of cytotoxicity. Because of its rapid bactericidal activity, that is mediated by its interaction with microbial membranes, it is difficult for bacteria and fungi to develop resistance to this dAMP.

As noted above, RP557 possesses broad-spectrum antimicrobial activity against multiple bacterial and fungal strains. Representative bacterial and fungal strains RP557 MIC values include: MRSA 1556, 8 µg/mL; MRSA 1756, 8 µg/mL; *S. aureus* 29213, 8 µg/mL; *S. epidermidis* 35984, 4 µg/mL; MDR SE 700578, 2 µg/mL; MRSE 51625, 4 µg/mL; Mycobacteroides abscessus 19977, 4 µg/mL; *P. aeruginosa* PA14, 2 µg/mL; *A. baumannii* ATCC BAA1605, 8 µg/mL; *Klebsiella pneumoniae* BAA1705, 8 µg/mL; *A. fumigatus* 18-25, 32 µg/mL; Mucor 17-102, 32 µg/mL; and *Fusarium sp*. 12-22, 32 µg/mL.

The *in vitro* activity of RP557 was further evaluated against *S. aureus* (methicillin-susceptible and methicillin-resistant; MSSA, and MRSA, respectively) that are resistant to ampicillin, lincomycin, erythromycin, mupirocin, fusidic acid, and bacitracin (**Table 1**).

Table 1. Minimal Inhibitory Concentrations (MIC) Data for RP557 and Comparator Drugs

MMX No.[1]	Phenotype	MIC (µg/mL)						
		RP557	Ampicillin	Lincomycin	Erythromycin	Mupirocin	Fusidic Acid[2]	Bacitracin
1013	MRSA; $MUP^{R}ERY^{R}FUS^{R}$	4	>32	32	>32	>256	8	>32

Table 1. Minimal Inhibitory Concentrations (MIC) Data for RP557 and Comparator Drugs								
MMX No.[1]	Phenotype	MIC (µg/mL)						
		RP557	Ampicillin	Lincomycin	Erythromycin	Mupirocin	Fusidic Acid[2]	Bacitracin
1004	MRSA; FUS^R	4	32	2	0.5	<0.25	16	>32
5698	MRSA; ERY^R	4	>32	4	>32	<0.25	0.5	>32
5699	MRSA; ERY^R	2	>32	2	>32	<0.25	0.25	>32
6311	MRSA; ERY^R	4*	>32	>32	>32	<0.25	0.5	>32
7779	MRSA; MUP^RERY^R	4	>32	32	>32	>256	0.25	>32
7782	MRSA; MUP^RERY^R	4*	32	>32	>32	>256	0.25	>32
1016	MRSA; FUS^R	4*	>32	2	0.5	<0.25	32	>32
8845	MRSA; MUP^RERY^R	2	>32	>32	>32	>256	0.25	>32
9202	MRSA; MUP^RERY^R	2	>32	>32	>32	>256	0.25	>32
0100	MSSA; ATCC 29213; QC	4	2	2	1	<0.25	0.25	>32
7684	MSSA; ERY^R	2*	0.25	2	>32	<0.25	0.25	16
8835	MSSA; ERY^R	4	32	2	>32	<0.25	0.5	>32
8836	MSSA; ERY^RFUS^R	4	8	4	>32	<0.25	1	>32
8837	MSSA; ERY^RFUS^R	8*	>32	32	>32	32	4	>32
3109	MSSA; ERY^R	4*	4	>32	>32	<0.25	0.25	>32
3248	MSSA; ERY^R	2	32	>32	>32	<0.25	0.25	32
3256	MSSA	4*	32	2	0.5	0.5	0.5	>32
3860	MSSA; ERY^R	4*	0.5	>32	>32	<0.25	0.5	>32
3866	MSSA; ERY^R	2	4	>32	>32	<0.25	0.25	>32
3885	MSSA	4	8	2	0.12	<0.25	0.25	16

[1]Studies performed by Micromyx, LLC, Kalamazoo, MI, US.
[2]Fusidic acid breakpoint by EUCAST >1 µg/mL is resistant. No CLSI breakpoints.
*Next dilution lower was a +/- with >80% reduction in growth, small pin point cell button.

It should be noted that in the phenotypes that have demonstrated resistance to the comparator antibiotics, RP557 was effective in inhibiting the growth of the resistant strains.

In addition to MIC assays, the MBC (Minimum Bactericidal Concentration) of RP557 was measured for several phenotypes. The minimal bactericidal concentration (MBC) and MBC:MIC ratios are listed in **Table 2**; lower ratios of 1 to 4 are indicative of bactericidal activity. RP557 possesses MBC:MIC ratios of ≤4 against all but one *S. aureus* isolate (*S. aureus* MMX 3860) underscoring RP557's microcidal mode-of-action.

Table 2. RP557 Minimal Inhibitory, Minimal Bactericidal Concentrations and MBC:MIC Results				
MMX No.	Phenotype	RP557 MIC or MBC (µg/ml)		
		MIC	MBC	MBC:MIC
1013	MRSA; $MUP^RERY^RFUS^R$	4	16	4
1004	MRSA; FUS^R	4	4	1
5698	MRSA; ERY^R	4	4	1
5699	MRSA; ERY^R	2	4	2
6311	MRSA; ERY^R	4*	4	4
7779	MRSA; MUP^R ERY^R	4	4	1
7782	MRSA; MUP^R ERY^R	4*	4	1
1016	MRSA; FUS^R	4*	4	1
8845	MRSA; MUP^R ERY^R	2	2	1
9202	MRSA; MUP^R ERY^R	2	2	1
0100	MSSA; ATCC 29213; QC	4	4	1
7684	MSSA; ERY^R	2*	2	1

Table 2. RP557 Minimal Inhibitory, Minimal Bactericidal Concentrations and MBC:MIC Results				
8835	MSSA; ERY^R	4	4	1
8836	MSSA; ERY^R FUS^R	4	4	1
8837	MSSA; ERY^R FUS^R	8*	8	1
3109	MSSA; ERY^R	4*	4	4
3248	MSSA; ERY^R	2	2	1
3256	MSSA	4*	4	1
3860	MSSA; ERY^R	4*	32	8
3866	MSSA; ERY^R	2	2	1
3885	MSSA	4	4	1
*Next dilution lower was a +/- with >80% reduction in growth, small pin point cell button.				

Bactericidal activity with limited cytotoxicity: AMP development has been hampered by unwanted toxicity to mammalian cells. RP557 exhibited limited, to no toxicity against human keratinocytes **(Figure 2).**

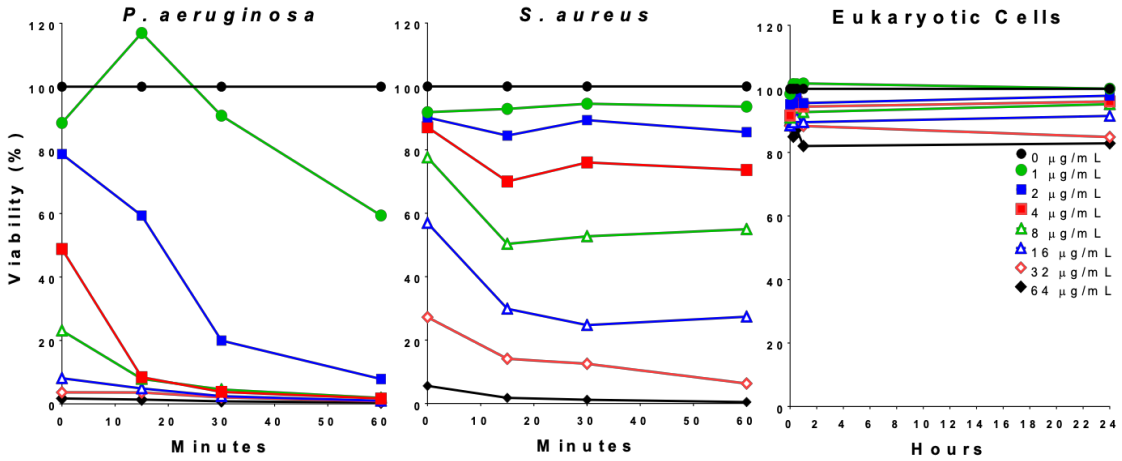


Figure 2. **RP557 rapidly eradicates *P. aeruginosa* and *S. aureus* with no cytotoxicity to mammalian cells**. Cell viability was performed using bioluminescent strains of *P. aeruginosa* 19660, *S. aureus* 49525 & L929 fibroblast cells. Data represents the mean of 3 measurements.

Following 8 hr incubation of keratinocytes with dAMPs, RP557 exhibited limited cytotoxicity with no cell killing observed at 256 µg/mL and 83.2% viability at the relatively high concentration of 512 ug/mL. In addition, there was no hemolysis of red blood cells incubated with RP557 at 128ug/ml **(Figure 3**).

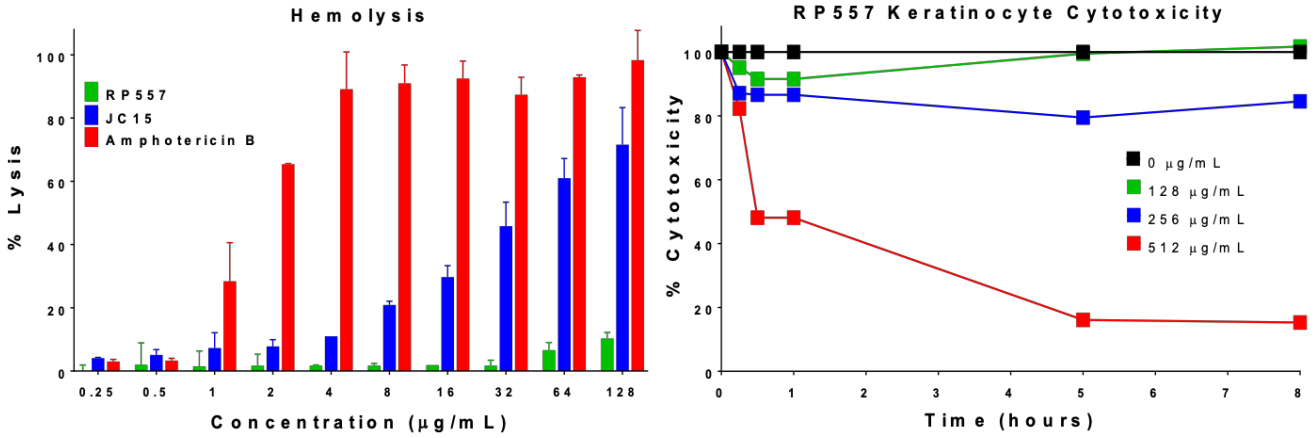


Figure 3. RP557 has limited toxicity against human red blood cells or cultured keratinocytes.

Major Task 3 – Third iterative round of dAMP synthesis and In Vitro Assays. Subtask 1c: Bacterial and fungal biofilm eradication testing of the most active dAMPs from above. We have continued to evaluate RP557 and RP554 for their ability to kill fungi in both planktonic and biofilm form.

Four dAMPs were evaluated for their antifungal activity against Fluconazole sensitive and Fluconazole resistant strains of Candidia. *Candida* clinical isolates were obtained from the California Institute for Medical Research (CIMR), San Jose, CA. The isolates selected for dAMP evaluation included *C. albicans* (5/10), *C. glabrata* (5/4), *C. tropicalis* (5/2), *C. parapsilosis* (5/1), and *C. krusei* (0/3), with the fractions given representing susceptible, S and resistant R (S/R), responses to fluconazole. Other *Candida* clinical isolates were tested against fluconazole for comparative purposes.

Minimal Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) Determination
Inhibition of planktonic growth (MIC) was assessed for the 46 clinical isolates using the broth macrodilution method in RPMI1640 using the Clinical and Laboratory Standards Institute (CLSI) methodology (CLSI, 2008), with breakpoints used for fluconazole as per CLSI. The range of concentrations tested included 0.5–32 µg/mL comprising 2-fold serial dilutions **(Table 3)**.

Table 3. MIC values of dAMPs against Fluconazole resistant and sensitive strains of Candidia.

***Candida* Species**	**RP504**	**RP554**	**RP556**	**RP557**
Res. *C. albicans*	16(1), 32(2), >32(7)	16(4), 32(5), >32(1)	8(2), 16(5), 32(3)	16(2), 32(5), >32(3)
Sus. *C. albicans*	32(5)	16(4), 32(1)	16(4), 32(1)	32(4), >32(1)
Res. *C. glabrata*	>32(4)	32(3), >32(1)	32(4)	32(2), >32(2)
Sus. *C. glabrata*	32(1), >32(4)	32(5)	16(2), 32(3)	32(4), >32(1)
Res. *C. tropicalis*	4(2)	2(1), 4(1)	2(2)	4(2)
Sus. *C. tropicalis*	4(4), 8(1)	2(1), 4(4)	2(4), 4(1)	4(3), 8(2)
Res. *C. parapsilosis*	>32	32	32	>32
Sus. *C. parapsilosis*	8(1), 32(1), >32(3)	2(1), 4(1), 16(3)	8(2), 32(3)	8(1), 32(1), >32(3)
Res. *C. krusei*	>32(3)	16(2), 32(1)	8(1), 16(2)	16(1), 32(2)
Sus. *C. kefyr*	4(1), 8(1)	4(2)	2(1), 4(1)	4(1), 8(1)
Sus. *C. lusitaniae*	4	2	8	8
Sus. *C. dubliniensis*	32	8	8	16
Sus. *C. sphaerica*	16	8	4	8
Sus. *C. famata*	8	4	16	16

Inhibition of planktonic growth was assessed at the California Institute for Medical Research, San Jose, CA using current CLSI methodology (CLSI, 2008). Numbers in parenthesis indicate the number of strains with the MICs shown.

Biofilm Inactivation. Bacteria and fungi encased in biofilm are highly resistant to the host innate immune mechanisms and to antibiotic treatment. When biofilm is combined with the increased presence of antibiotic resistant strains, the treatment of infected patients is confounded. RP557 completely eradicates MRSA in both newly formed and robust biofilm **(Figure 4)**.

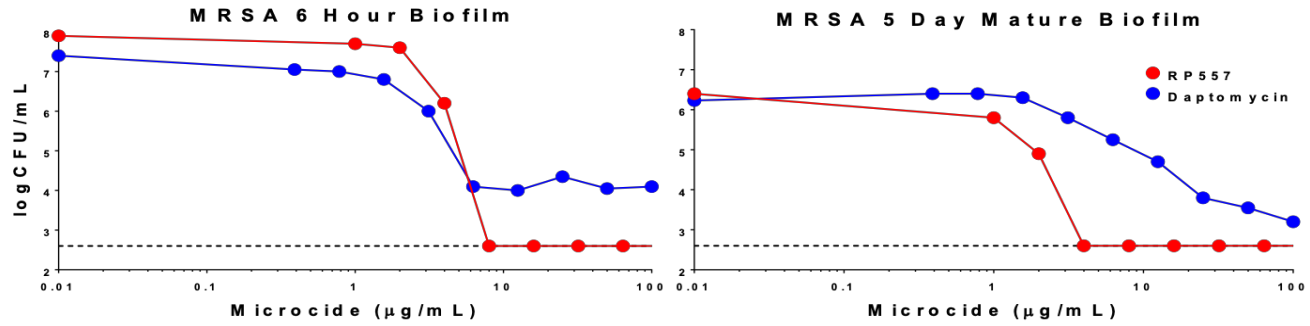


Figure 4. RP557 eradicates bacteria in both preformed and mature MRSA biofilm.

RP557 had EC_{50s} of 4.21 and 2.12ug/ml for preformed and mature biofilm respectively. By contrast Daptomycin was able to reduce preformed MRSA in biofilm by 50% with an EC_{50} of 4.1ug/ml. However, the concentration needed to reduce MRSA in mature biofilm was an order of magnitude higher at approximately 100ug/ml.

S. epidermis (Staphylococcus epidermis) is a major nosocomial pathogen and is among the most prevalent bacteria in human skin and mucous membranes. It presents a major problem in the treatment of biofilm infections, including biofilm on devices. RP557 is able to eradicate S. epidermis in biofilm **Figure 5**.

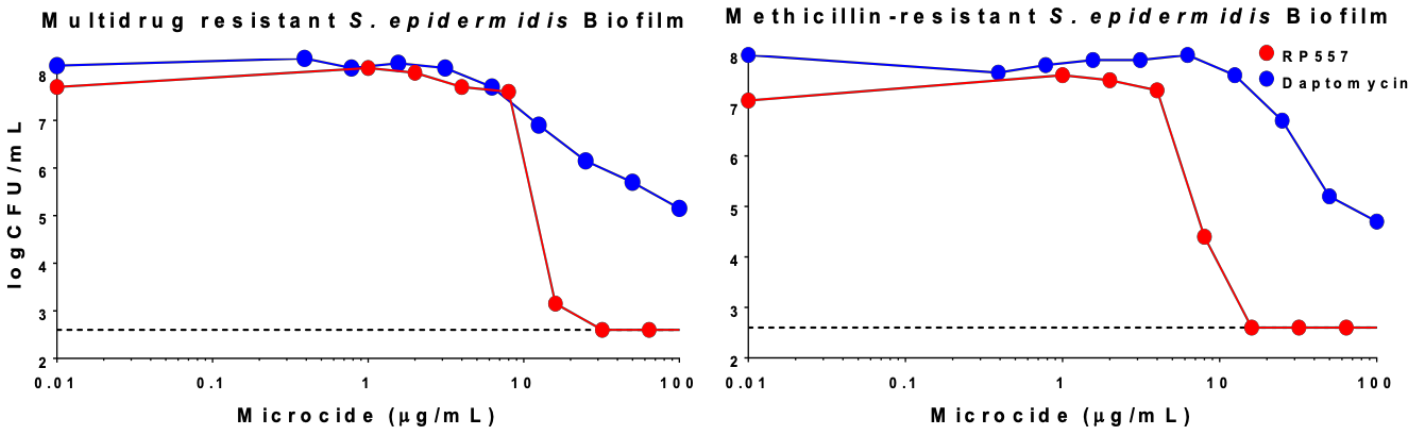


Figure 5. RP557 completely eradicates both MDR and MRSA resistant S. epidermis in Biofilm.
The EC_{50s} for MDR and MRSA were 7.72 and 9.31 ug/ml respectively while Daptomycin exhibited a mild effect. At 100 ug/ml Daptomycin reduced MDR S. epidermis biofilm by 37% and MRSA resistant S. epidermis biofilm by 41%.

Fungal Biofilm Inhibition

Candida albicans 17-88, $5x10^5$ cell/ml, was allowed to form biofilm for 16 hours, washed three times with 200 µl PBS, and subsequently challenged immediately, or following 24 hours, with the test articles to assess effects on biofilm formation or preformed biofilm inhibition, respectively. (Nazik et al., 2017). The dAMPs or fluconazole were diluted in RPMI1640 with final well concentration being 64, 32, 16 and 8 µg/ml and compared to control wells containing only RPMI. Each condition contained 3 replicates. Following a total incubation time of 40 hours, the wells were washed three times with 200 µl PBS, and then 200 µl XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) [phenyl-amino) carbonyl]-2H)) and menadione solution were added to each well. The plates were incubated at 37°C for 2 hours and absorbance read, and data presented, at 490 nm for assessment of inhibition of metabolism. The endpoint was a statistically significant reduction, compared to an untreated concurrent control.

The Biofilm Activity Quotients in **Table 4** demonstrate that the dAMPs are more effective than Fluconazole at inhibiting both the formation of biofilm and fungal growth in biofilm.

Table 4. Susceptibility of Planktonic Yeasts, Biofilm Formation, and Preformed Biofilm (*C. albicans* isolate 17-88) to the inhibitory effects of dAMPs and Fluconazole.

Agent	MIC (µg/mL)	Biofilm Formation MIC Inhibition* (µg/mL)	Biofilm Formation Activity Quotient#	Preformed Biofilm MIC Inhibition* (µg/mL)	Preformed Biofilm Activity Quotient#
Fluconazole	1	64	64	64	64
RP504	32	>64	>2	64	2
RP554	16	32	2	8	0.5
RP556	16	32	2	32	2
RP557	32	16	0.5	4	0.125

*lowest concentration with statistically significant inhibition

#ratio of biofilm activity endpoint, statistically significant difference, to MIC.

Fungal Biofilm Eradication. Fungal infections are frequently found in war wounds and biofilm is a frequent driver of pathogenicity. RP557 effectively kills the fungus C. albicans (Candida albicans) in its preformed biofilm

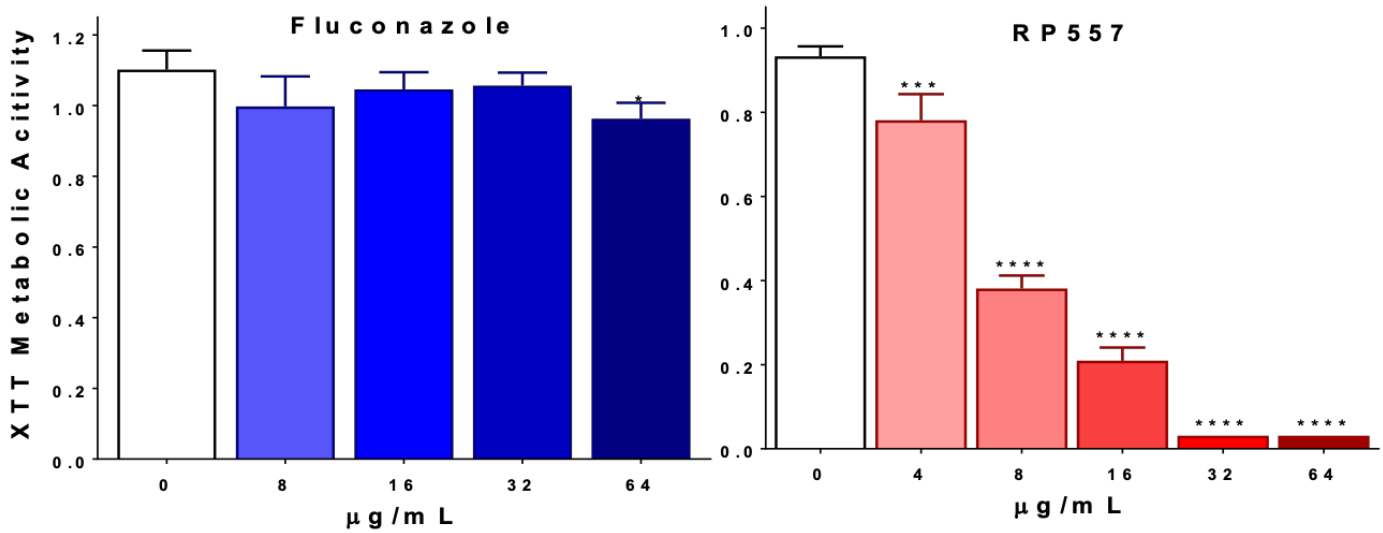


Figure 6.

Figure 6. RP557 effectively kills C. albicans in preformed biofilm while Fluconazole is ineffective. Data represents the mean of three measurements with statistical significance compared to vehicle control assays.

Significance was determined by one-way ANOVA followed by Dunnett's Test (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

Direct Cell Wall Perturbation: Scanning electron microscopy on planktonic and on preformed biofilm *C. albicans* indicated that dAMP mediated fungal damage was via membrane perturbation, whereas fluconazole was fungistatic. Control planktonic *C. albicans* appeared as well-rounded and intact yeasts with prominent bud-scars; whereas fluconazole-treated cells had scattered irregular areas across the cell surface, reflecting some compromised cell membranes and walls. The membrane surfaces of dAMP treated planktonic cells, in contrast, were dramatically irregular and roughened which increased in severity with concentration, reflecting increased cell wall damage (cells treated with a representative dAMP, RP554, are shown in **Fig. 7**).

In biofilms, fluconazole-treated cells showed only coating with a film-like or granular substance, likely residual extracellular matrix. The surfaces of the *Candida* cells within the biofilm, containing some pseudohyphal or hyphal cells, became roughened, corrugated and flattened upon treatment with RP554, increasing with increasing concentrations, indicating severe cell wall damage, and implying cellular perturbation via membrane disruption.

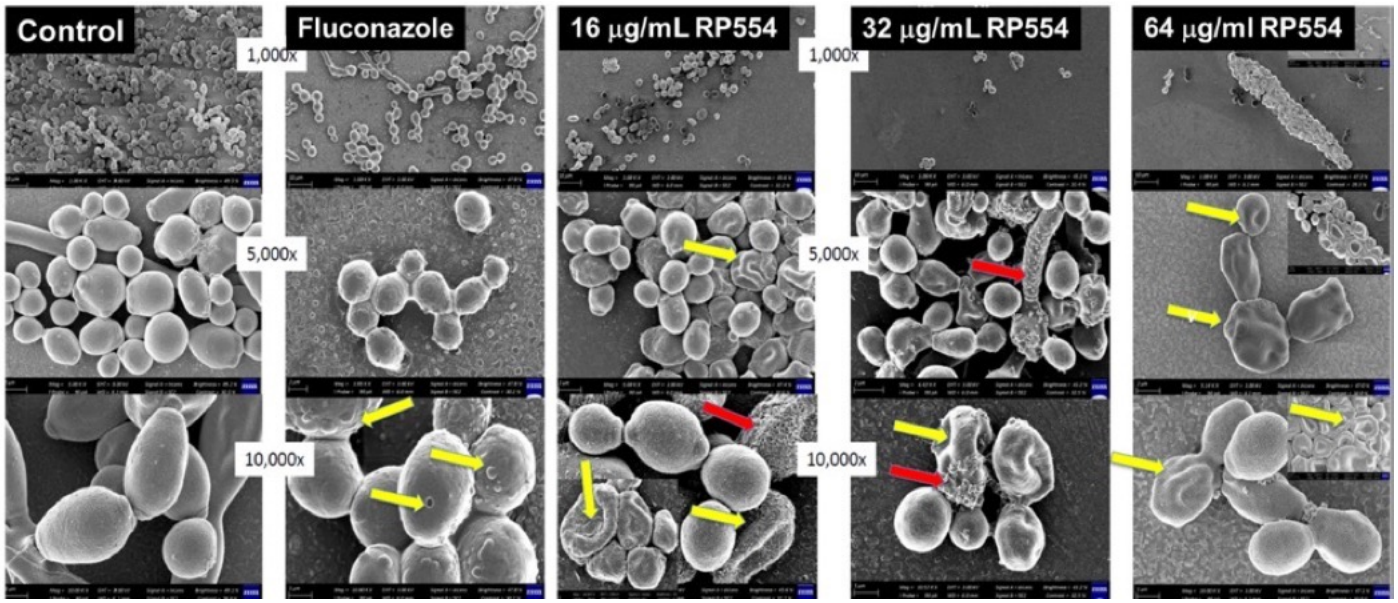


Figure 7. Damage of *C. albicans* biofilm by RP554. Scanning electron micrographs of *C. albicans* 17-88 biofilm incubated with test article for 24 hours: control (no treatment); 64 µg/mL fluconazole; 16, 32, and 64 µg/mL RP554. The yellow arrows indicate cell wall indentations and cell membrane damage, red arrows indicate surface coating with extracellular matrix residue.

Pathogens Do Not Develop Resistance To AMPs. The increasing emergence of antibiotic resistant microbial strains highlights the need for innovative alternatives that have limited susceptibility to the mechanisms that confer antibiotic resistance. The Time-Kill assays shown in Figure 2 indicate that RP557 rapidly disrupts membrane function thereby killing potentially infective organisms. This rapid bactericidal effect provides little opportunity to develop membrane structures that are not recognized by a dAMP. However, the ability of RP557 to induce resistance was evaluated by repeatedly exposing either S. aureus or P. aeruginosa to increasing concentrations of the peptide **Figure 8**.

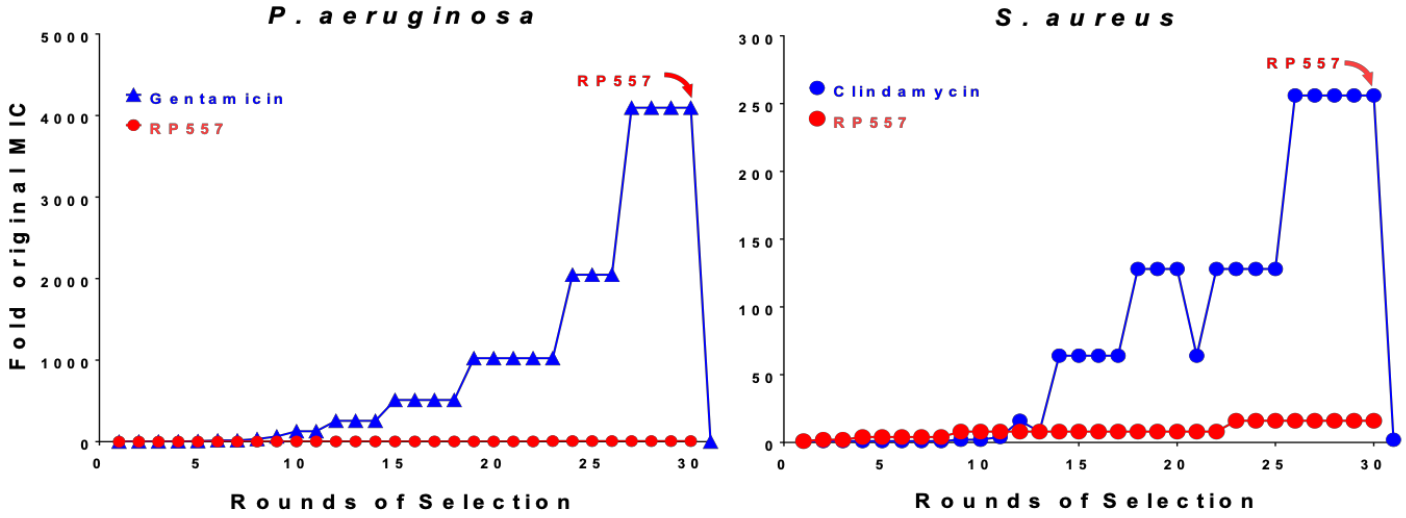


Figure 8. Pathogens did not develop resistance to RP557. Sub-inhibitory concentrations of RP557, Gentamicin or Clindamycin were incubated for 24 hours with P. aeruginosa 27853 or S. aureus 29213. Bacteria growing in the highest concentration were re-passaged to fresh dilutions containing sub-MIC levels of each component for 30 consecutive passages.

Neither P. aeruginosa or S.aureus developed resistance to RP557 while each strain developed resistance to Gentamicin or Clindamycin respectively. At the 30th passage of the resistant strains they were cultured with RP557. The MIC value of resistant strains to RP557 was the same as the original MIC value, indicating that the induced resistance to standard antibiotics was not transferred to the dAMP mechanism for killing the bacterial strain.

Bactericidal Activity *In Vivo*. RP557 dose-dependently reduced both P. aeruginosa and S.aureus in a polymicrobial porcine burn wound infection **Figure 9**. Full thickness wounds were created on the backs of anesthetized pigs with a heated brass rod and a trephine. This was followed by infecting the wound with a 2:2:1 mixture of S.aureus 6538, P.aeruginosa (porcine isolate) and fusobacterium ssp. After 3 hours, 0, 0.1, 0.2, 1 or 2% solutions of RP557 in 3% hydroxypropyl methyl cellulose 4000cps, 30% propylene glycol in water was applied to wounds. After 24 hours, wounds were sampled by punch biopsy and bacterial counts, expressed as log CFU/g tissue (Colony Forming Units).

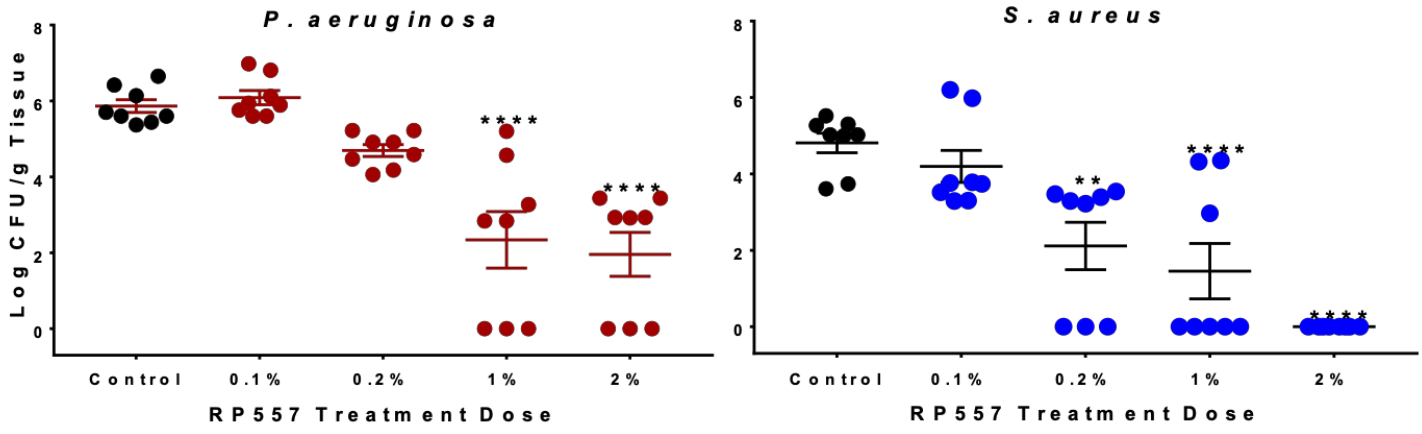


Figure 9. Dose-response to RP557. Data is expressed as the mean of 8 replicates ± SEM. Statistical significance was determined by one-way ANOVA followed by Dunnett's test (** $p < 0.001$, **** $p < 0.0001$).

As discussed above, biofilm is physical barrier that hinders the interaction of antimicrobials and antibiotics with microbes. It is generally recognized that bacteria in wounds will have formed a biofilm by 24 hours post infection. We have thus conducted a study with treatment beginning 24 hours after a polymicrobial infections of porcine burn wounds. A 2% (20mg/ml) solution of RP557 was applied to wounds 24 hours post infection. Biopsies were taken for bacterial counts 24 hours after treatment **(Figure 10)**.

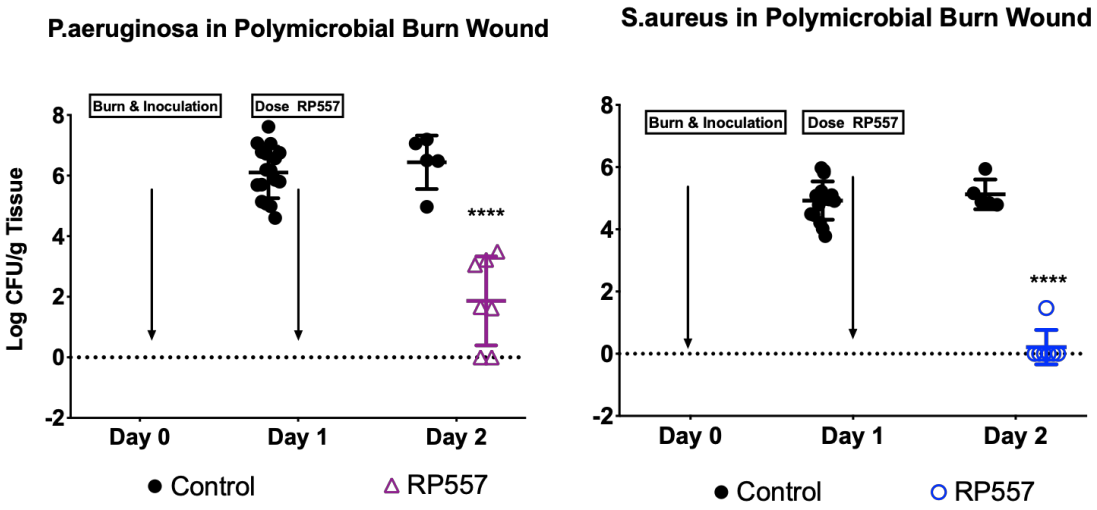


Figure 10. RP557 Treatment of 24 Hour Polymicrobial Infection. Data are expressed as the mean of 5 to 8 replicates ± SEM. Statistical significance was determined by one-way ANOVA followed by Dunnett's test (**** $p < 0.0001$).

The statistically significant reductions in bacterial infections are based on the fact that on Day 2 the P.aeruginosa control was measured at Log 6.44 CFU/g tissues and RP557 reduced this level to Log 1.86 CFU/g tissue. This is a reduction of over 4.5 Logs. In similar action, the S.aureus levels were reduced from Log 5.12 CFU/g control tissue to Log 0.21 CFU/g RP557 treated tissue – a reduction of over 4.9 Logs. These are reductions of over 99.99% of infecting bacteria.

The data in **Figure 11** (above) demonstrates that 24 hours after the application of RP557, the dAMP has been able to penetrate the biofilm and kill bacteria residing therein. Our *in vitro* time-kill data has demonstrated that in a planktonic culture bacterial killing occurs upon exposure to RP557 see **Figure 2**. To determine the onset and progression of bacterial killing *in vivo* biofilm, biopsies were taken 30min, 180 min and 24 hours after RP557 (2%) was applied to burn wound infected for 24 hours. Within 30min of application, (**Figure 11** below) RP557 has significantly reduced the CFU/g tissue of both P.aeruginosa and S.aureus. The peptide continues to kill the infecting bacteria over the next 24 hours.

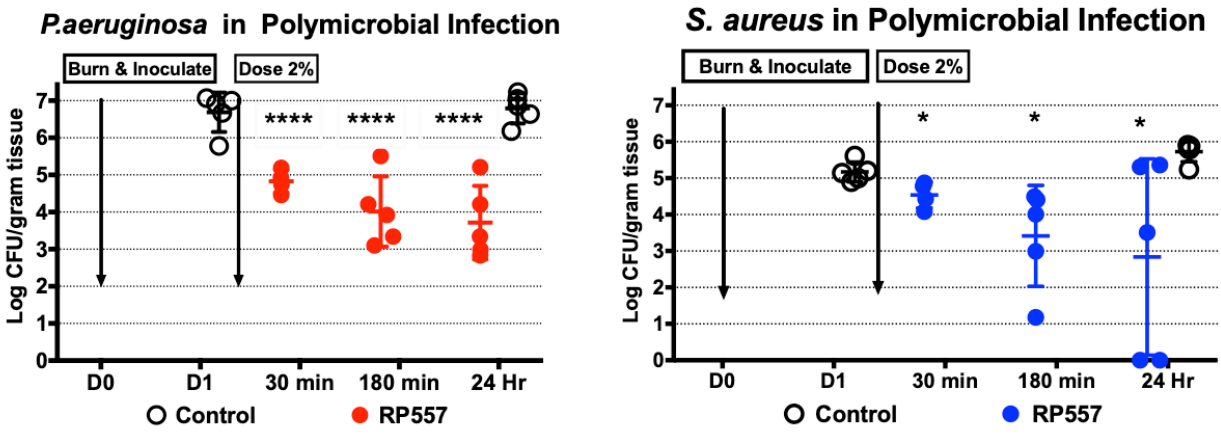


Figure 11. RP557 Treatment of 24 Hour Polymicrobial Infection. Data are expressed as the mean of 5 replicates ± SEM. Statistical significance was determined by one-way ANOVA followed by Dunnett's test (* $p<0.02$) (**** $p < 0.0001$).

While the data in Figure 10 indicates that RP557 is able to penetrate into the wounded tissue to the level of the biofilm, it does not provide data on the actual location of the biofilm or how far into the tissue the drug may be found. To determine the extent to which dAMPs interact with the biofilm and surrounding tissues an assay was developed by ImaBiotech to detect the level of RP557 in biopsies taken from the study described in Figure 11. This study is part of work associated with SOW **Major Task 4 – Selection of Peptides for QMSI, and formulation.** Subtask 2b: Select the three most active peptides for tissue penetration studies by QMSI (Quantitative Mass Spectrometry Imaging) and have them synthesized for formulation studies.

1. Brief Study Design: MS method development was undertaken to select the most appropriate MALDI matrix for detection of RP557. RP557 solutions were spotted onto indium tin oxide coated slides and the following MALDI matrices were added: 2,5-dihydroxybenzoic acid (DHB; positive mode); α-cyano-4-hydroxycinnamic acid (CHCA; positive mode); 1,5-diaminonaphtalene (1,5-DAN; negative mode); and 9-aminoacridine (9-AA; negative mode). The slides were dried under vacuum at room temperature. Each spot was analyzed with a 7T-MALDI-FTICR (Solarix, Bruker Daltonics, Billerica, MA) in full-scan mode.

Table 5. RP557 MALDI Matrix Signal-to-Noise Ratios

Matrix	Signal-to-Noise Ratio
DHB	4336.9
CHCA	1849.0
1,5-DAN	ND
9-AA	ND
ND, not detected. n > 30 spectra	

The best signal for RP557 was obtained with the DHB matrix in positive mode (**Table 5**).

The RP557 major ionized form was $[M+H]^+$ ion form. Sodium and potassium adducted molecules were detected at lower intensity level than the $[M+H]^+$ ion form. Optimization of the DHB matrix warrants investigation to allow the best detection of RP557.

The most appropriate solvent to use for MALDI matrix dissolution for the best detection of RP557 was then investigated. RP557 solutions were spotted onto control skin sections. The slides were dried under vacuum at room temperature. Each spot was analyzed with 7T-MALDI-FTICR. DHB 40mg/mL in methanol/H_2O 1% TFA 1:1 (v/v) allowed the best detection of RP557 on control porcine skin sections (**Table 6**).

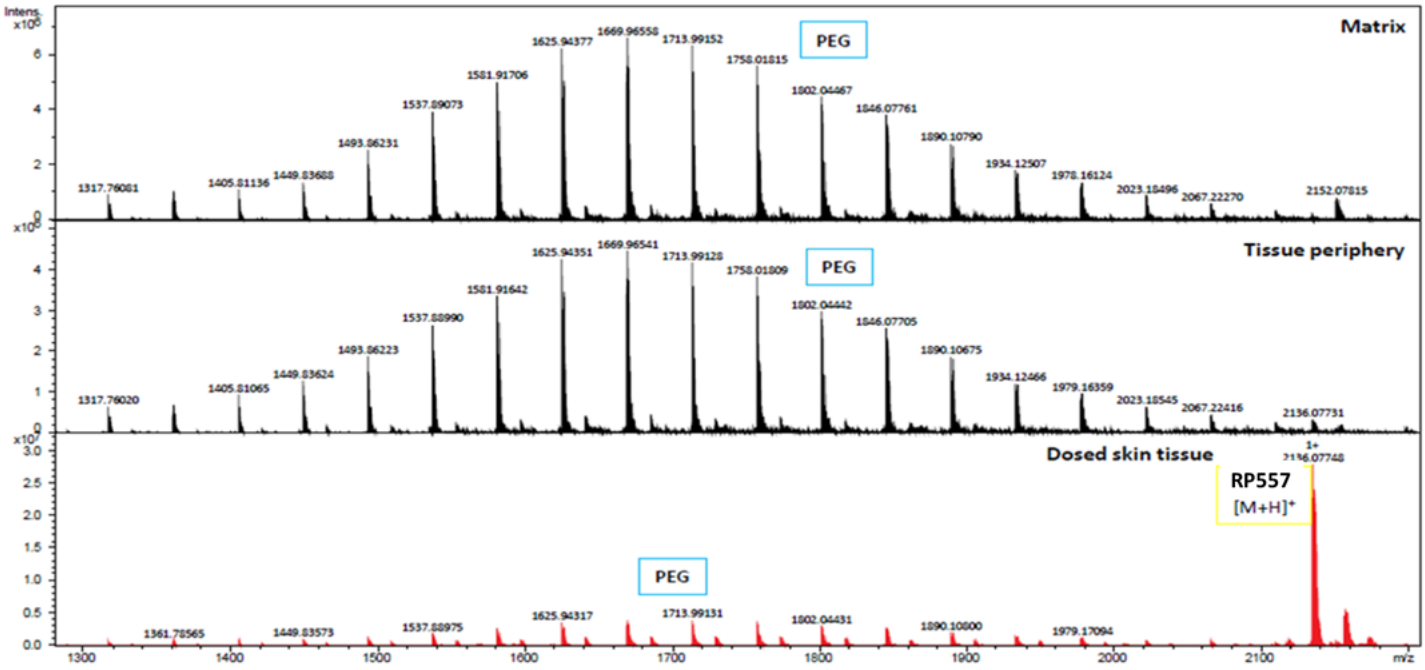
Table 6. MS RP557 Signal Intensity Spotted onto Control Skin

Solution Tested	RP557 Intensity
DHB 1:1 Methanol/H_2O (v/v) + 0.1% TFA	1.3+007
DHB 1:1 Methanol/H_2O (v/v) + 1% TFA	**1.4+007**
DHB 7:3 Methanol/H_2O (v/v) + 0.1% TFA	5.6+006
DHB 7:3 Methanol/H_2O (v/v) + 1% TFA	1.2+007

RP557 was then evaluated in dosed porcine skin samples using the optimized matrix and solvent conditions. DHB MALDI matrix at 40 mg/mL in methanol/H2O 1% TFA 1:1 (v/v) was sprayed onto 10 µm tissue sections from control and dosed porcine samples (2%) using an automatic sprayer device. Tissue sections were imaged by 7T MALDI-FTICR at 300 µm spatial resolution. The data was analyzed with Multimaging v1.1.6 software (ImaBiotech, Lille, France). RP557 was detected in the skin section of the dosed pig (**Figure 12**). Polyethylene glycol (PEG) was also detected during the analysis of the RP557 dosed skin tissue sample. The PEG signal did not interfere with the signal of the $[M+H]^+$ ion for RP557.

Figure 12: Direct detection of RP557 in topically dosed porcine skin by 7T-MALDI-FTICR Mass Spectrometry. The PEG fragmentation pattern from the vehicle formulation does not overlap with the signal $[M+H]^+$ ion of RP557. The skin sample was taken 3 hr following 2% RP557 topical application in pigs. The molecular formula of RP557 is $C_{99}H_{151}N_{27}O_{18}S_4$ with an associated molecular weight of 2134.0608 amu yielding a molecular ion $[M+H]^+$ of 2135.0686 amu and $[M-H]^-$ of 2133.0529 amu.

With the establishment of an assay that can detect RP557 in pig skin, we are prepared to assay infected wound biopsies that have been treated with RP557. These tissues are being prepared for this analysis by Dr. Robert Christy's lab (USAISR) in accordance with the CRADA agreement between his lab and Riptide Bioscience, Inc. From these studies we will be able to determine tissue penetration by RP557.



Fungicidal Activity *In Vivo*. As a model for evaluating the effectiveness of RP557 against fungal infections in vivo, we chose a validated model of vulvovaginal candidiasis. The results demonstrate that RP557 was effective in eradicating the fungus C. albicans in a rodent model of vulvovaginal candidiasis **Figure 13**. The topical application of RP557 2% was as efficacious as topical Miconazol, whereas oral Fluconazole was ineffective.

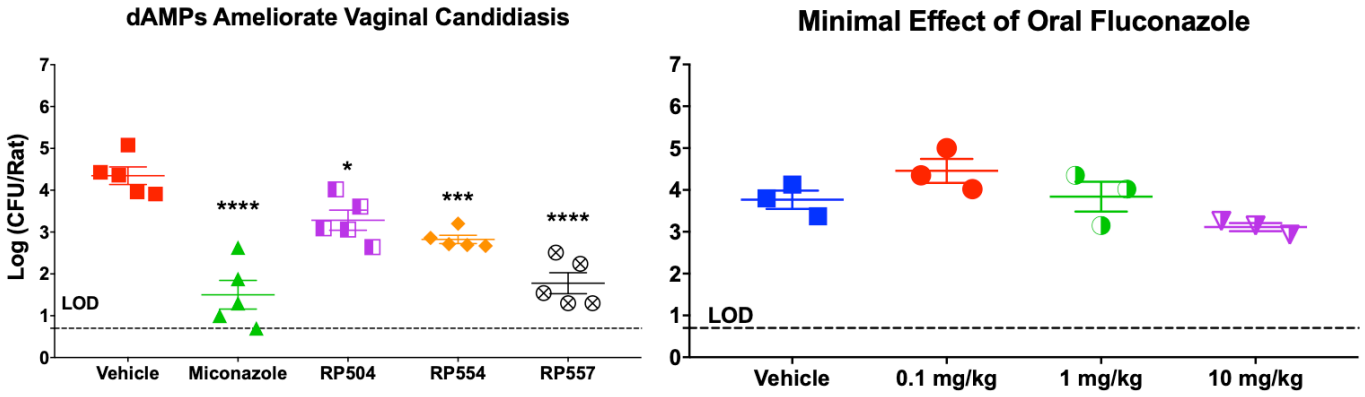


Figure 13. **Effects of topical Miconazole and RP557 and Oral Fluconazole on a rat model of vulvovaginal candidiasis.** On day 0 rats were inoculated intravaginally with C. albicans (ATCC 44858) at $1.46x10^7$ CFU/rat.

A 2% solution of each dAMP and Miconazole were injected intravaginally twice daily at 8hour intervals beginning 48 hours post infection. Dosing continued for 3 days. CFU levels were measured on day 5 with LPD of 0.7 CFU/rat. Significant differences were calculated vs Vehicle by ANOVA followed by Dunnett's test (* $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$).

Conclusions:

- **dAMPs disrupt membrane function**. The rapid dose-response demonstrated in the time-kill studies suggests that the dHDPs are killing bacteria by disrupting essential membrane functions **(Figure 2)**. It is highly unlikely that bacteria will develop resistance to this mechanism of bactericidal activity.
- **RP557 has minimal cytotoxicity**. 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 **(Figures 2 & 3)**.
- **RP557 does not induce bacterial resistance.** The resistance to antibiotics is not transferred to resistance to RP557 **(Tables 1, 2, 3, Figures 4 & 5)**.
- **RP557 significantly reduces *in vivo* polymicrobial infections (Figures 9, 10 &11).** Most wounds have polymicrobial infections with biofilm. This is a clinically relevant model and result.
- **RP557 effectively kills Fluconazole resistant C. albicans (Table 3).**
- **RP557** has both bactericidal and fungicidal activity **(Figures 2 & 7)** .
- **RP557 reduces C. albicans infections (Figure 13).** Many wounds have both bacterial and fungal infections. Thus, RP557 has the potential to be a single agent for the treatment of wounds with multiple microbial infections.
- **RP557 can be detected ` in topically dosed porcine skin by 7T-MALDI-FTICR Mass Spectrometry.**

4. Impact

RP557 has been identified as a compound that has the potential be a single agent for the treatment of wounds infected with a wide range of bacteria and fungi.

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. ImaBiotech has been contracted to assay RP557 in biopsies from treated burn wounds. Dr. Robert Christy's lab at USAISR will perform cytokine assays and histological analysis of burn wound biopsies treated with RP557.

Additional studies are needed to assess the in vivo toxicity and potential for systemic absorption. These studies will be conducted during the next year.

6. Products

Reviewed data for JPC2 meeting May 2018
Poster #1401 presentation at MHSRS 2018

7. Participants and other Collaborating Organizations

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8. Special Reporting Requirements: None

9. Appendix: None