Novel Bisphosphocin Nu-3 Demonstrates Rapid Killing of Bacteria-Encased in Biofilm *In vitro*

D. Akiyoshi[1], J. Dilo[1], P. DiTullio[2]
1. Tufts Cummings School of Veterinary Medicine, N. Grafton, MA;
2 Lakewood-Amedex Inc, Sarasota, FL

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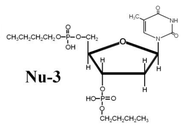
ABSTRACT

Background: Bacterial biofilms pose a significant treatment challenge to traditional therapies because it enables the bacteria to persist in a dormant or slow growth phase. Biofilms are increasingly becoming associated with chronic infections and implantable device failure as well as resistance of bacteria to conventional antibiotics, highlighting the need for more effective therapeutics. Nu-3, a member of a novel class of extremely broad-spectrum antimicrobials, was evaluated for its ability to rapidly kill biofilm-encased bacteria *in vitro*.


Nu-3

Methods: The bactericidal activity of Nu-3 against biofilm-encased bacterial strains of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Staphylococcus epidermidis* using a time kill assay to assess the post-antibiotic effect (PAE). Bacteria were grown 24 hours in tryptic soy broth with 1% dextrose (TSBD) in sets of four borosilicate glass tubes to allow formation of the biofilm on the tube wall. The medium was carefully removed using a pipet and the tubes treated with 170U/ml Nu-3 for 10 min and 30 min, or sterile saline. Following the room temperature incubation, the tubes were washed with sterile saline. TSBD was added to all four tubes and the tubes were incubated at 37°C for 24 hours without shaking. After 24 hour incubation, the cultures were visually examined for growth and appropriate dilutions were made and aliquots plated in onto TS agar plates to enumerate colonies.

Results: Nu-3 exhibited a rapid a rapid bactericidal effect on biofilm-encased bacteria with a 100% kill of all four bacterial strains observed at 170U/ml and exposure time of 10 minutes. This result further supports experimental data showing Nu-3 is directly bactericidal through a mechanism of action involving depolarization of the cell membrane and is in contrast to most traditional antibiotics.

Conclusion: Nu-3 displayed rapid bactericidal activity against biofilm-encased bacteria highlighting its potential as a new topical antimicrobial therapy for infected wounds or prophylactic treatment prior surgical closure or implantation of medical devices.

ACKNOWLEDGEMENTS

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INTRODUCTION

Bisphosphocins™ are a new class of broad spectrum antimicrobials that possess a unique mechanism of action involving membrane depolarization which leads to rapid cell death. The bisphosphocin Nu-3 is being developed as an intravenously administered treatment for severe bacterial infections such as complicated urinary tract infections. The aim of the present studies was to evaluate the ability of Nu-3 to kill bacteria encased in biofilm formed on the wall of a borosilicate glass tube. Biofilms represent a challenge for traditional antibiotics because the bacteria become dormant or slow growing which makes them better able to resist the affect of antibiotics. Scientific research has linked biofilm formation with many types of chronic infections, implant failure, and treatment failure making it a growing healthcare concern. Bisphosphocins™ provide a potential therapeutic solution to this problem as they are directly bactericidal and thus their activity is not reduced when bacteria become sessile or slow growing. Early experiments showing an increased sensitivity of stationary bacteria to Nu-3 laid the ground work for the current set of experiments evaluating its activity against several biofilm forming strains.

MATERIALS AND METHODS

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): Overnight bacterial cultures (tryptic soy broth (TSB); 37C) were subcultured 1:100 into fresh TSB and grown for 2-3 hours. The cultures were diluted to a concentration of ~1 x 10e7 cfu/ml. Nu-3 was added to cultures containing 5 x 10e5 cfu/ml to final concentrations between 21.25 to 255 Units/ml and incubated at 37°C with shaking for 24 hrs. Control tubes with untreated bacteria and medium-only were included. After 24 hrs, cultures were diluted and plated onto tryptic soy agar plates to quantify the number of colony forming units (cfu's). After 24 hrs at 37°C, the number of cfu's were determined and from these results, the MIC and MBC were determined for each bacterial isolate.

Time Kill Protocol: Overnight bacterial cultures (tryptic soy broth (TSB); 37C) were subcultured 1:100 into fresh TSB and grown for 2-3 hours. The cultures were diluted to a concentration of 2 x 10e6 cfu/ml, centrifuged and resuspended in sterile saline (0.85% NaCl) at 1 x 10e5 cfu/ml. Nu-3 in sterile saline was added to a final concentration of 3.4 to 170U/ml to the bacteria and the bacteria/Nu-3 mixture incubated at room temperature. At time points ranging from 5 to 60 minutes, aliquots were removed, diluted and plated onto TS agar plates for quantitation of the number of colony forming units (cfu's). After 24 hrs at 37°C, the number of cfu's were determined and the Nu-3 concentrations and times resulting in 100% kill were determined.

Establishment and Staining of Biofilm: Bacteria were streaked onto TS agar plates from glycerol stocks and grown overnight at 37°C. An overnight culture (5 ml, tryptic soy broth with 1% dextrose (TSBD)) was started from a single colony and grown overnight at 37°C. The overnight culture was diluted 1:100 with fresh TSBD and 2.5 ml was added to 3 glass tubes per bacteria. All tubes were incubated at 37°C without shaking to allow biofilm formation. A control tube containing medium-only was included.

After 24-hour incubation, the medium was carefully removed from all tubes using a pipette. To two of these tubes, Nu-3 (2.5 ml; 10 mg/ml in sterile saline) was added. To the third bacteria tube and medium-only tubes, sterile saline (2.5 ml) was added. The tubes were incubated at room temperature. After a 10 min or 30 min Nu-3 incubation, the tubes were washed with sterile saline to remove residual Nu-3 (3 washes of 5 ml each). The untreated bacterial tube was washed twice with 3 ml sterile saline. TSBD (2.5 ml) was added to all four tubes (2 Nu-3-treated, 1 untreated and 1 medium-only) and the tubes were incubated at 37°C for 24 hours without shaking. After 24-hour incubation, the cultures were visually examined for growth and appropriate dilutions were made and aliquots plated in duplicate onto TS agar plates. The plates were incubated at 37°C overnight and colonies enumerated.

After the cultures were diluted and plated, the cultures were carefully removed from the tubes using a pipet. The tubes were rinsed several times with water and then stained with crystal violet.

RESULTS

Crystal Violet Staining of Biofilms at 24hrs and 48hrs

A. baumannii BAA-1605		*S. epidermidis* PCI1200		*S. epidermidis* 35984	
24hrs	48hrs	24hrs	48hrs	24hrs	48hrs
					

Nu-3 Activity is both Concentration and Time Dependent

Bacterial Strain	Nu-3 Concentration		
	10.625 (U/ml)	85 (U/ml)	170 (U/ml)
A. baumannii BAA-1605	30 min	10 min	5 min

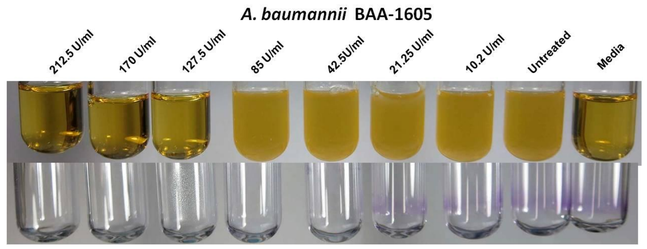
Activity of Nu-3 Against Bacteria Established in Biofilm

Strain	*A. baumannii* BAA-1605		*S. epidermidis* 35984		*S. epidermidis PCI 1200*	
Biofilm Producer	Low		High		Low	
Treatment	Untreated	Nu-3 170U/ml	Untreated	Nu-3 170U/ml	Untreated	Nu-3 170U/ml
Exposure Time	10min	10min	10min	10min	10min	10min
24hr CFU Count	2 x 10^9	N.G	2.7 x 10^7	N.G.	5.2 x 10^0	N.G.
Crystal Violet Stain						

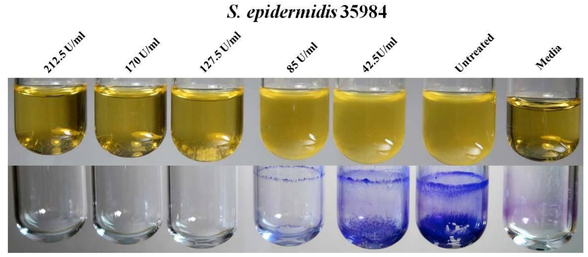
N.G. = No Growth

Effect of Bisphosphocin™ Nu3 on Biofilm Formation

A. baumannii BAA-1605



S. epidermidis 35984



Planktonic MIC and MBC Values for Nu-3

Bacterial Strain	MIC (U/ml)	MBC (U/ml)
A. baumannii BAA-1605	127.5	127.5
S. epidermidis 35984	170	170

CONCLUSION

- Bisphosphocin™ Nu-3 displayed potent bactericidal activity against *A. baumannii* and *S. epidermidis* 35984 encased in a biofilm layer formed on the surface of a borosilicate glass tube at a concentration of 170U/ml and exposure time of 10 minutes.

- The MBC of Nu-3 for these two strains was equivalent whether the bacteria were growing planktonically or established in a biofilm layer. In addition, the data indicate that the bactericidal activity is not affected whether it is a low biofilm producer, *A. baumannii* or a high producer, *S. epidermidis*.

- Time kill studies with Nu-3 demonstrate a rapid kill rate of both *Staphylococcus epidermidis* 35984 and *Acinetobacter baumannii* with 100% kill of 1 x 10^5 CFU at a concentration of 85U/ml and exposure time of 5 minutes. This rapid kill rate has been shown to be both dose and exposure time dependent in contrast to traditional antibiotics (data not shown) that function through an inhibitory mechanism over a prolonged time period.

- Bisphosphocin™ Nu-3 holds significant potential for the treatment of wide range of serious bacterial infections due to its broad spectrum of activity, unique mode of action, rapid killing, and ability to kill bacteria in a biofilm layer.