Antimicrobial Efficacy of a Nitric Oxide-Releasing Drug Candidate *in vitro* and *in vivo* Utilizing an Infected Porcine Partial Thickness Burn Wound Model

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ABSTRACT

Background: Burn injuries sustained from explosions and fires account for 5-10% of combat casualties and multidrug-resistant infections are growing at an alarming rate. Silver-containing products such as silver sulfadiazine (SSD) have long been considered the standard topical therapy for patients with burns; however, the efficacy of these treatments for improving mortality, wound healing, and decreasing infection rates has been questioned and novel treatments are urgently needed. Exogenous nitric oxide has been investigated as an antimicrobial agent due to its broad-spectrum activity and ability to attack multiple biochemically essential targets, making the development of resistance unlikely. Novan's proprietary technology stably stores the gaseous species as an engineered macromolecule allowing control of nitric oxide release. **Methods:** The antimicrobial efficacy of NVN4428 and SSD against the common wound pathogens: *Acinetobacter baumannii,* methicillin-resistant *Staphylococcus aureus*, and *Candida albicans* was assessed in vitro via time-kill assays. Antimicrobial efficacy against biofilm-embedded species was determined *in vitro* using the MBEC (Innovotech) device. The antimicrobial efficacy of topical treatments was assessed *in vivo* using a porcine deep partial thickness burn wound infection model at 4 and 7 days post-wounding. **Results:** While both NVN4428 and SSD demonstrated antimicrobial efficacy *in vitro* under planktonic conditions, decreased efficacy was observed when *in vitro* biofilm cultures were challenged with SSD and NVN4428. In a biofilm burn wound model, NVN4428 treatment reduced *A. baumannii* by >5-logs compared to the <2-log reduction observed with SSD treatment on day 4. Similarly, the superiority of NVN4428 over SSD treatment was observed for all tested organisms. **Conclusions:** These studies demonstrate that NVN4428 has superior *in vivo* antimicrobial efficacy over SSD against all tested organisms.

MATERIALS & METHODS

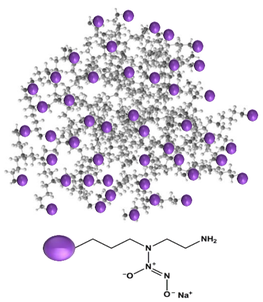
Time-Kill Assays: Time-Kill assays were performed in accordance with the ASTM E2315 Standard. Varying concentrations of antimicrobial agents (silver sulfadiazine or NVN4428) were incubated with each microorganism (10^6 CFU/ml) in Tris buffer (100 mM, pH 7.5) at 37°C. Untreated cultures were included as controls. At 1 and 4 hours of incubation samples were taken and serial dilutions were plated to determine the number of viable bacteria.

Minimum Biofilm Eradication Concentration: The minimum biofilm eradication concentrations (MBECs) for *A. baumannii, C. albicans,* and *S. aureus* were determined using the MBEC 96-well assay plate (Innovotech) routinely used for high throughput screening of biofilm-embedded bacteria. Biofilms were grown for 24 hrs at 37°C prior to challenge with NVN4428 and silver sulfadiazine. Biofilms were challenged with antimicrobial agents for 18 hours and then sonicated for 25 minutes in recovery media to disperse the biofilm bacterial for serial dilution. Recovered biofilms were grown for 18 hrs at 37°C and bacterial viability was subsequently determined by MTT assay. Concentrations completely inhibiting growth of recovered bacteria are reported.

Deep Partial Thickness Wound Study: Three specific pathogen-free pigs (Looper Farms, NC) per microorganism were anesthetized and 51 rectangular wounds (10 mm ×7 mm × 0.5 mm deep) were made to the paravertebral and thoracic area with an elecrokeratome. Wounds were separated by 15 mm of unwounded skin and individually dressed. Eight wounds were randomly assigned to each treatment group. After creation of burns, 25µl of *A. baumannii* (AB 09-001*), Methicillin Resistant *S. aureus* (MRSA USA300) *and C. albicans* (CA 09-024*) was use to inoculate each wound by scrubbing (10^6 CFU/ml) inoculums into each wound with a teflon spatula (30 seconds). All wounds were covered individually with a polyurethane film dressing (Tegaderm). The bacterial biofilms were allowed to form for 2 days prior to treatment. Treatment groups were treated with ~200mg of test article and spread out to cover the wound and surrounding unwounded area with a sterile spatula and covered with film dressing. At the assessment time, the bacteria from 4 wounds per treatment group were recovered in 1 ml of neutralization solution and serially diluted. Serial dilutions were subsequently plated on selective media and incubated for 24 hours at 37°C prior to enumeration of viable colonies. Colonies were counted and the colony forming units per ml (CFU/ml), Log CFU/ml, mean Log CFU/ml and standard deviation calculated. A one-way analysis of variance (ANOVA) was used for statistical analysis. A p value of less than 0.05 was considered significant.

RESULTS

NVN4428



Nitric Oxide-Releasing Macromolecule

Stable formulation development under Army Contract # W81XWH-11-C-0029

PhoGel48

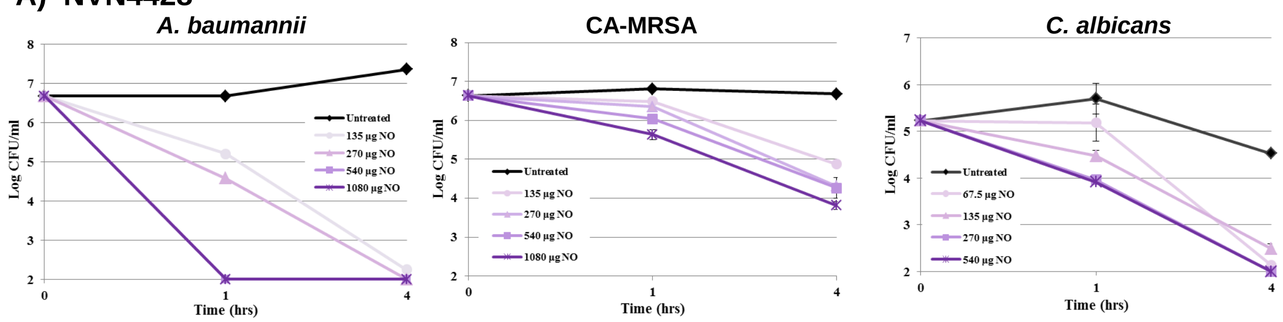
1:1

NVN4428 Ointment : Hydrogel

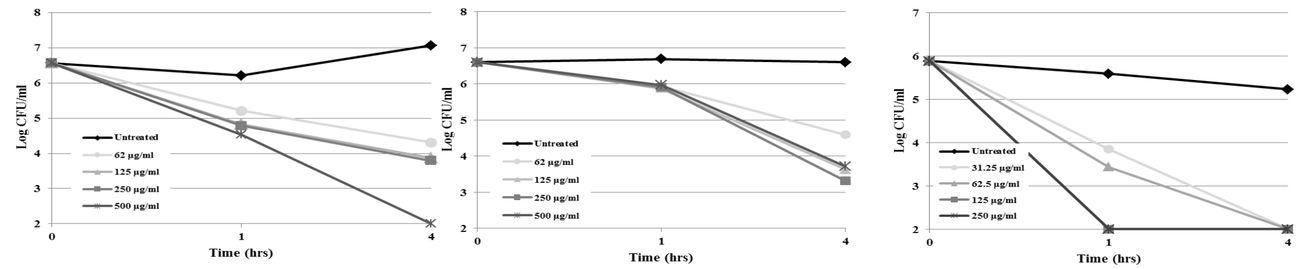
Figure 1. In Vitro and In Vivo Bactericidal Activity Against Multidrug-Resistant Organisms.

Multidrug-resistant *Acinetobacter baumannii*, community-acquired methicillin-resistant *Staphylococcus aureus,* and *Candida albicans* were treated *in vitro* with NVN4428 (A) or Silver Sulfadiazine (B). In vivo efficacy of topical formulations was assessed in a porcine partial thickness infected wound model (C). ‡ statistically different from vehicle at corresponding timepoint. *statistically different from silver at designated timepoint.

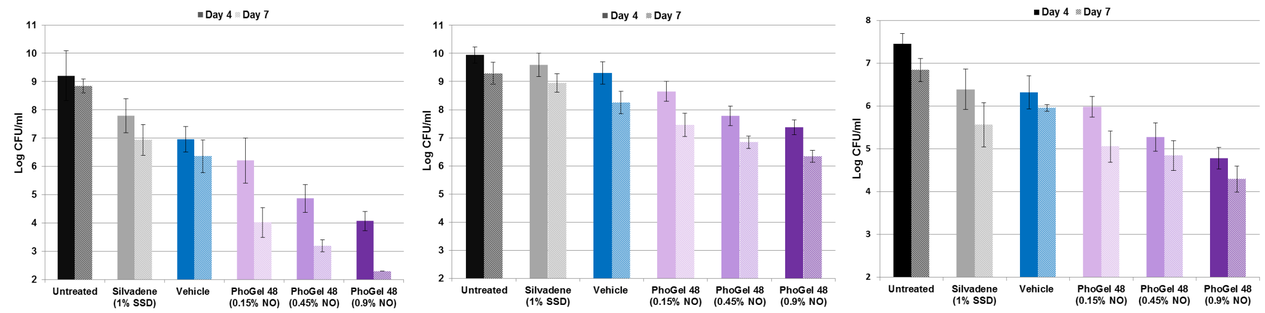
A) NVN4428

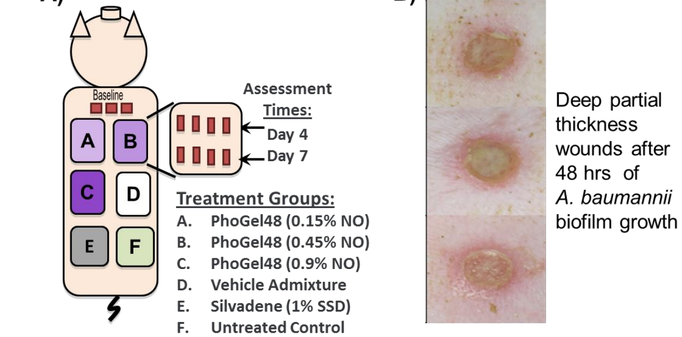


B) Silver Sulfadiazine



C) In Vivo Activity





Deep partial thickness wounds after 48 hrs of *A. baumannii* biofilm growth

Table 1. Bactericidal Efficacy Against Planktonic and Biofilm-Embedded Multidrug-Resistant Microorganisms.

Microorganisms were grown either planktonically or as biofilms and exposed to drug substance (NVN4428 or silver sulfadiazine). The concentration of antimicrobial agent required to achieve bactericidal efficacy is reported.

Multidrug-resistant Organism	Silver Sulfadiazine Concentration for 3-log planktonic reduction at 4hrs	MBEC Silver Sulfadiazine	NVN4428 Concentration for 3-log planktonic reduction at 4 hrs	MBEC NVN4428
A. baumannii	500 µg/ml	250-500 µg/ml	135 µg NO	1080 µg NO
CA-MRSA	500 µg/ml	500 µg/ml	*Not achieved	540 µg NO
C. albicans	31.25 µg/ml	250-500 µg/ml	270 µg NO	1080 µg NO

- **NVN4428 demonstrated rapid *in vitro* bactericidal activity against *A. baumannii* with comparable activity to silver sulfadiazine against CA-MRSA and *C. albicans*.**
- **The *in vitro* biofilm assay results were not predictive of the *in vivo* pharmacological comparison between silver sulfadiazine and nitric oxide-releasing NVN4428.**

- **PhoGel48 demonstrated a dose responsive reduction in bacterial load that was significantly more efficacious compared to Silvadene in a porcine partial thickness infected burn wound model.**
- **After 5 once daily treatments (Day 7), the reduction in bacterial load with 0.9% PhoGel48 compared to Silvadene was:**
  - **99.99% more effective against *A. baumannii***
  - **99% more effective against CA-MRSA**
  - **90% more effective against *C. albicans***

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