Susceptibility of *Staphylococcus aureus* to Nitric Oxide-Releasing Macromolecules and Assessment of Resistance

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ABSTRACT

Background: Nitric oxide is a promising antimicrobial agent due to its ability to attack multiple biochemically essential bacterial targets, producing broad-spectrum activity with a low propensity for resistance. Novan's Nitricil technology solves the previous issues in delivering exogenous nitric oxide by stably storing the gaseous species as an engineered macromolecule. The characteristics of these compounds can be controlled to tune the level of nitric oxide storage, the rate of nitric oxide release, and the molecule size to target nitric oxide delivery. **Methods:** Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of two Nitricil compounds (NVN4428 and NVN1000) were determined for 43 *Staphylococcus aureus* isolates using the broth microdilution method. NVN4428 and NVN1000 were selected to determine the effect of nitric oxide release rate on antimicrobial activity. NVN4428 has a much slower release rate compared to NVN1000 ($t_{1/2}$ = 58 min vs. 2.3 min). The macromolecules were loaded with similar amounts of nitric oxide (4.5 µmol nitric oxide/mg NVN4428 vs. 5 µmol nitric oxide/mg NVN1000). To identify potential mutants with reduced susceptibility, a subset of *S. aureus* strains were passed serially for 30 days at sub-MIC concentrations. **Results:** *S. aureus* MICs for NVN4428 (Range: 0.25-4 mg/ml; theoretical dose of 34-540 mg NO/ml) were 2-to 32-fold lower than MICs for NVN1000 (Range: 4-16 mg/ml; theoretical dose of 600-2400 mg NO/ml) for all strains tested. MBCs for NVN1000 matched the corresponding MICs while NVN4428 MBCs were typically two-fold higher than the established MICs. Exposure of *S. aureus* to bactericidal concentrations of NVN1000 or NVN4428 particles failed to produce spontaneous resistance mutants with reproducible increases in MIC or MBC values upon retesting. Serial passaging resulted in minimal (< four-fold) changes in the MICs of *S. aureus* strains. **Conclusions:** These data suggest that lower magnitude, more sustained release profiles of nitric oxide from NVN4428 provide greater antimicrobial activity against *S. aureus* than the high-burst, short half-life of nitric oxide release from NVN1000. Furthermore, *S. aureus* strains exhibit a low propensity of developing resistance to NVN1000 or NVN4428.

MATERIALS & METHODS

Minimum Inhibitory Concentration: MICs for *S. aureus* were determined according to the Clinical Laboratory Standards Institute (CLSI) guidelines for broth microdilution (CITE). MIC assays were performed in a 96-well format (100 µl/ well) with the following modifications: The CLSI-recommended growth media, Cation-Adjusted Mueller Hinton Broth (CAMHB), was made with 100 mM Tris buffer to maintain culture pH upon the introduction of Nitricil particles. Additionally, all MIC assays were performed at 37°C for 18 hours. Bacterial viability was assessed by MTT assay.

Minimum Bactericidal Concentration:

30 day serial passage: Based upon the previously determined NVN4428 MIC values (Table 2), we selected six *S. aureus* strains for serial passage in the sustained Nitricil formulation (NVN4428), and also simultaneously passaged strains in ciprofloxacin for comparison (Figure 2). Bacteria were grown overnight on TSA and then resuspended in 100 mM Tris CAMHB to achieve a concentration of 10^8 CFU/ml. *S. aureus* suspensions were then added to serial dilutions of antimicrobials resulting in a 96-well plate containing 10^5 CFU/ml and two-fold dilutions of ciprofloxacin or NVN4428 encompassing the MIC values. Following a 24-hour incubation at 37°C, MIC values were recorded, and an aliquot from the well containing the highest antimicrobial concentration that supported bacterial growth was diluted to 2 x 10^5 CFU/ml and used to inoculate the subsequent serial passage plate. The entire process was repeated for 30 days.

RESULTS

Nitricil Technology

Figure 1. Structures of NVN1000 (A) and NVN4428 (B).

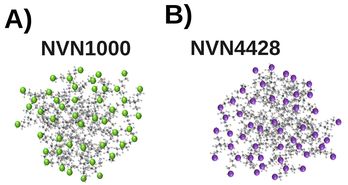


Figure 2. Nitric Oxide Release Profiles of Different Nitricil Compositions. Nitricil particles can be designed with different maximum instantaneous fluxes of nitric oxide **(A)** maintaining the same total amount of nitric oxide (t[NO]) released over time **(B)**.

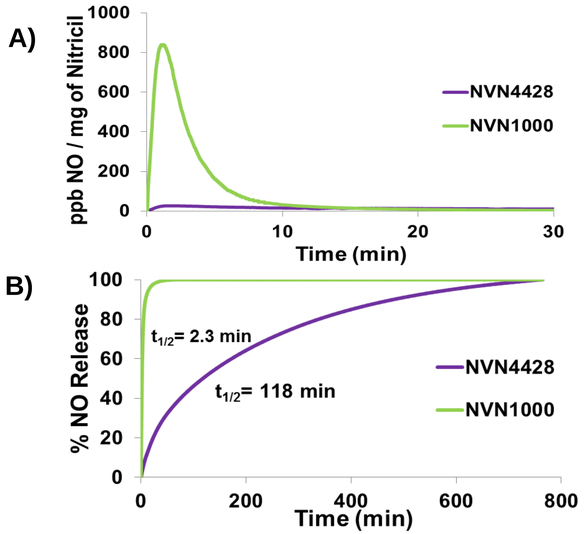


Figure 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for 45 *S. aureus* strains. *Values for NVN1000* ***(A)*** *and NVN4428* ***(B)****. Strains are designated according to Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) repository from which they were obtained.*

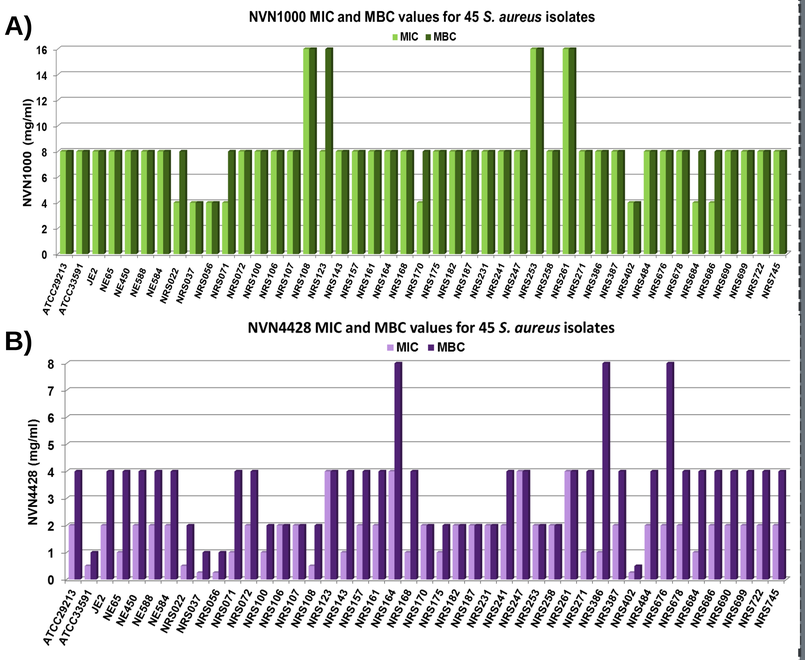
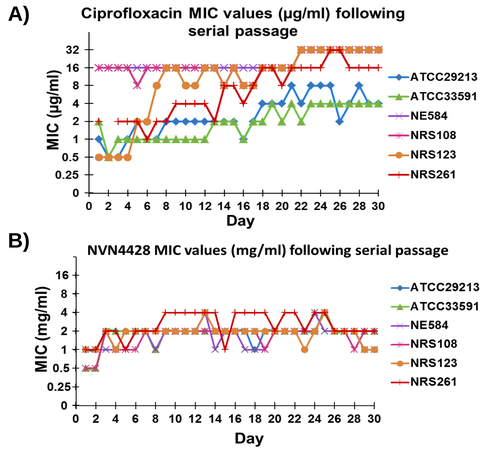


Table 1. Nitricil Susceptibility Testing of *S. aureus* isolates. *Concentrations at which 90% of the tested strains were susceptible (MIC/MBC/MBEC$_{90}$) are reported in terms of both particle weight along with the full range of values.*

S. aureus	**NVN1000**	**NVN4428**
MIC$_{90}$	**8 mg/ml** Range: 4-16 mg/ml	**2 mg/ml** Range: 0.25-4 mg/ml
MBC$_{90}$	**8 mg/ml** Range: 4-16 mg/ml	**4 mg/ml** Range: 1-8 mg/ml

RESULTS

Figure 4. 30 day serial passage of selected *S. aureus* isolates in Ciprofloxacin (A) and NVN4428 (B).



CONCLUSIONS

- *S. aureus* MIC values for NVN4428 were 2-to-32-fold lower than the MIC values using NVN1000.
- NVN1000 MBC values closely matched the previously determined MIC values, while NVN4428 MBC values were typically one-fold higher than the established MIC values.
- These data indicate that the lower more sustained release of nitric oxide from NVN4428 is more effective than the rapid high burst of nitric oxide release from NVN1000 at eradicating *S. aureus.*
- Following 30-day serial passage with NVN4428, minimal changes in the MIC values were observed for the six strains tested, whereas serial passage in Ciprofloxacin resulted in the development of resistant strains (MIC values > 4 µg/ml)
- Given these results, *S. aureus* strains have a limited propensity to develop resistance to Nitricil particles.

REFERENCES

1. Journal Article, *Name of Journal*
2. Journal Article, *Name of Journal*