Nitric Oxide is Bactericidal to the ESKAPE Pathogens: Time for a radical approach

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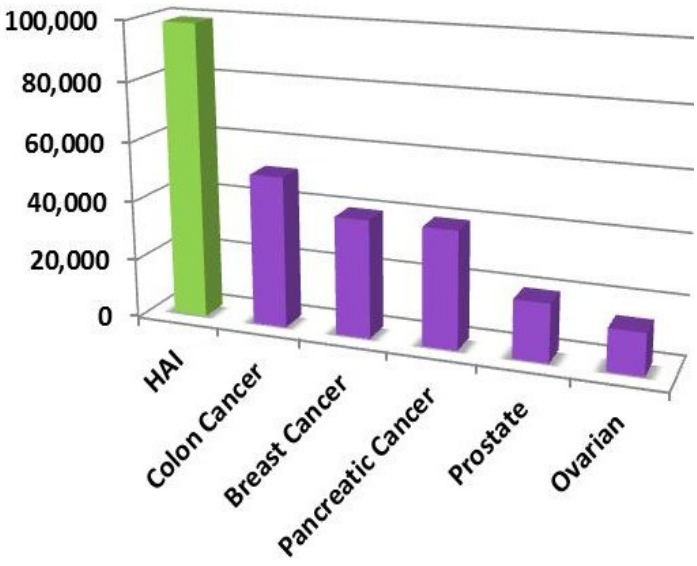
Infections caused by drug-resistant bacteria kill more Americans every year than colon and breast cancer combined (Figure 1). While the frequency of infections caused by antibiotic-resistant bacteria continues to rise, the number of antimicrobial drugs in development have steadily declined (Boucher 2009, 2013 and Taubes 2008). Antibiotic-resistant infections now cost the U.S. healthcare system an estimated $20-35 billion per year. Taken together, these statistics have resulted in what the Infectious Diseases Society of America has described as "an urgent, immediate need" for antibacterial agents that are effective against multi- and pan-resistant organisms (Boucher, 2009).

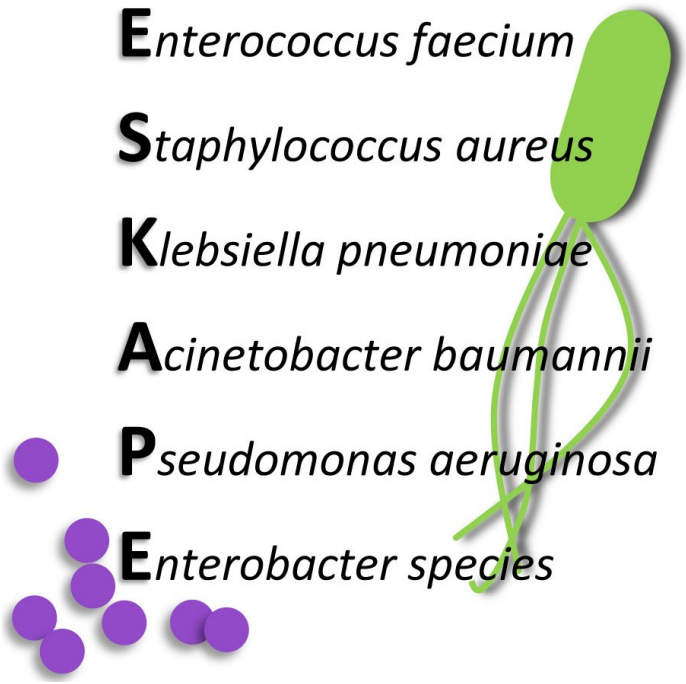
Figure 1. Estimated 2013 U.S. deaths from hospital-acquired infections (HAIs) in comparison to five of the most notable forms of cancer. (**Centers for Disease Control and Prevention, American Cancer Society 2013*)



ESKAPE Pathogens

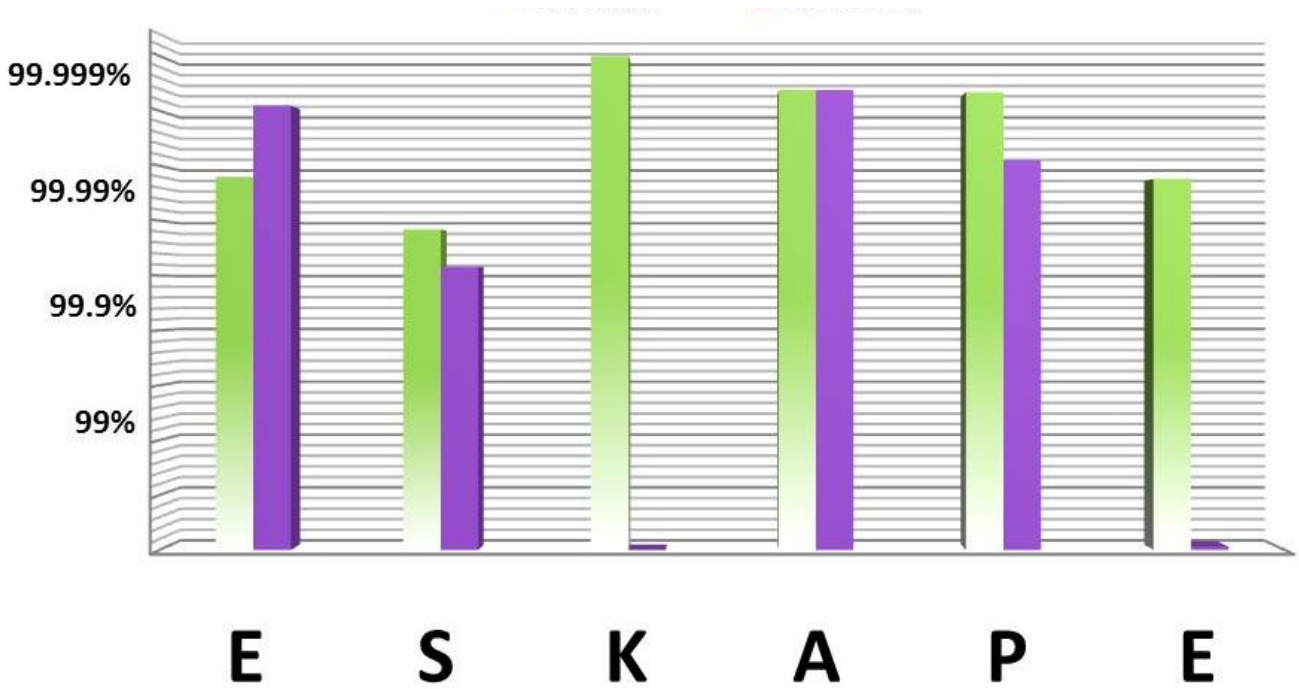
Over the last decade, the unrelenting proliferation of antibiotic-resistant bacteria has escalated, led predominantly by a formidable group of bugs designated as the "ESKAPE" pathogens (Figure 2). The ESKAPE pathogens are responsible for the majority of U.S. hospital infections, and presently "escape" the arsenal of currently available antibiotics through their evolutionary defense mechanisms (Rice, 2008 and Boucher, 2009). The number of fatalities (~19,000/year) in U.S. hospitals associated with methicillin-resistant *Staphylococcus aureus* (MRSA) now exceeds the number of deaths from HIV/AIDS (Klevens, 2006). While once only a threat to the hospitalized patient population, the ESKAPE pathogens, along with other drug resistant Gram-negative bacilli, are rising in prevalence among otherwise healthy nonhospitalized patients (Boucher 2013). Upwards of 50% of patients infected with extremely drug resistant (XDR) *Klebsiella* or XDR *Acinetobacter* will succumb to their infection despite treatment with last resort antibiotics (Boucher 2009 and Falagas 2005).

Figure 2. The ESKAPE Pathogens. *A group of Gram-negative and Gram-positive bacteria is responsible for the majority of HAIs with widespread antibiotic resistance.*



Nitric oxide therapy represents a radical approach that harnesses the body's natural defense mechanism to eliminate a broad spectrum of pathogens.

Figure 3. Percent killing of ESKAPE pathogens after 4 hours at a fixed dose of 4 mg/mL NVN1000 or NVN4428.



Nitric Oxide - A Radical Approach

Given this medical crisis, it is imperative that new therapeutic strategies for which it is difficult for pathogens to develop resistance, are developed. The development of bactericidal agents that kill bacteria, rather than inhibit bacterial growth, are necessary because the eradication of microorganisms restricts the development of bacterial resistance (Stratton, 2003). Nitric oxide is a naturally occurring free radical endogenously produced by the human body. As a key component of the innate immune response, nitric oxide is produced by macrophages and other inflammatory cells during infection (Alderton 2001, Tripathi 2007).

Bactericidal Activity of NVN1000 and NVN4428

In vitro time-kill studies were performed to determine the bactericidal efficacy of two nitric oxide releasing drug candidates (NVN1000 and NVNV4428) against the panantibiotic-resistant ESKAPE pathogens using a third party conducted time-kill assay. This method measures changes in a bacterial population of aerobic microorganisms within a specified time period when treated with antimicrobial agents. Bacteria were resuspended in Tris buffer to a standard starting inoculum of 10^6 CFU/ml and treated with varying concentrations of NVN1000 or NVN4428. Bacteria were removed and plated at the 1- and 4-hour time points to determine the percent killing. Initial inoculums left untreated were included as growth controls. The percentage of bacteria killed by 4 hours for each of the ESKAPE pathogens is shown in Figure 3. Specific ESKAPE pathogens included carbapenem-resistant *K. pneumoniae* NDM-1 resistance plasmid, vancomycin-resistant *E. faecalis,* ciprofloxacin-resistant *P. aeruginosa,* a community-acquired MRSA USA300 strain, and a multi-drug resistant strain of *A. baumannii.*

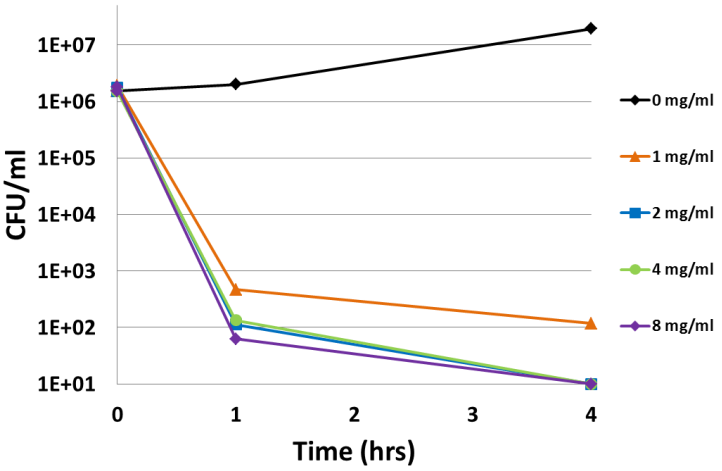
By 4 hours, greater than 99.9% of all ESKAPE pathogens were killed by NVN1000 treatment demonstrating the broad spectrum of NVN1000 activity (Figure 3). At the 4 hour time point, the slower nitric oxide releasing NVN4428 was as effective as NVN1000 at reducing the bacterial population of *S. aureus, A. baumannii, and P. aeruginosa.* However, for some Gram-negative pathogens (*K. pneumoniae, E. cloacae)* NVN4428 resulted in minimal bactericidal activity.

Just a Matter of Time

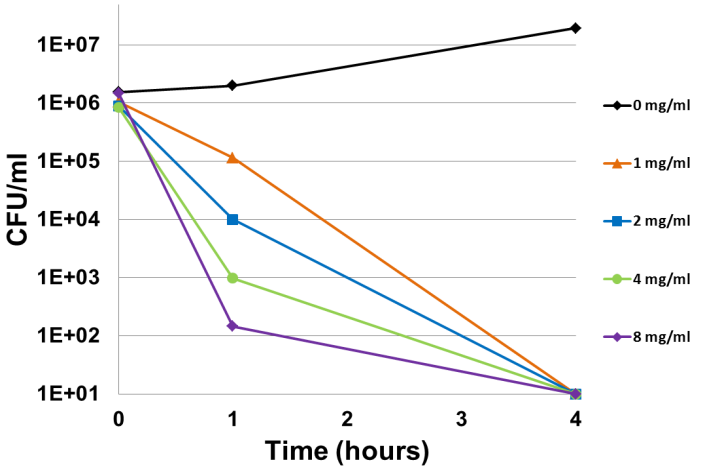
To demonstrate the effect of nitric oxide release kinetics on antimicrobial activity, time-kill curves for a specific organism, *A. baumannii,* are depicted in Figure 4. NVN1000 reduced the bacterial population by 99.9% in as little as one hour at all concentrations tested, while NVN4428 required concentrations of 4 mg/ml or higher to achieve a 99.9% reduction in the bacterial population at the same time point (Figure 4A and 4B). However, by 4 hours the effects of NVN1000 and NVN4428 on the *A. baumannii* population are nearly identical. After 4 hours of exposure, NVN1000 concentrations of 2 mg/ml or greater resulted in a 99.999% (5 -log) reduction. Similarly, all concentrations of NVN4428 achieved a 99.999% (5-log) reduction in the *A. baumannii* population following 4 hours of exposure.

Figure 4. Comparison of the bactericidal efficacy of NVN1000 (A) and NVN4428 (B) as a function of concentration against *Acinetobacter baumannii.*

A. NVN1000



B. NVN4428



These results highlight the effects of nitric oxide release rate on bactericidal efficacy as NVN1000 and NVN4428 have drastically different nitric oxide release profiles. NVN1000 delivers a rapid high burst of nitric oxide (Figure 5A, $t_{1/2}$= 2.3 min) while NVN4428 delivers a lower more sustained release (Figure 5A, $t_{1/2}$ =118 min). Thus, for *A. baumannii* a powerful short burst of nitric oxide results in rapid bactericidal activity (Figure 4A, 1 hr. time point). However, *A. baumannii* is also effectively reduced by lower levels of nitric oxide if these levels are maintained for a longer time period (Figure 4B, 4hr time point). After 4 hours of exposure, all concentrations of NVN4428 resulted in a 99.999% (5-log) reduction of the *A. baumannii* population.

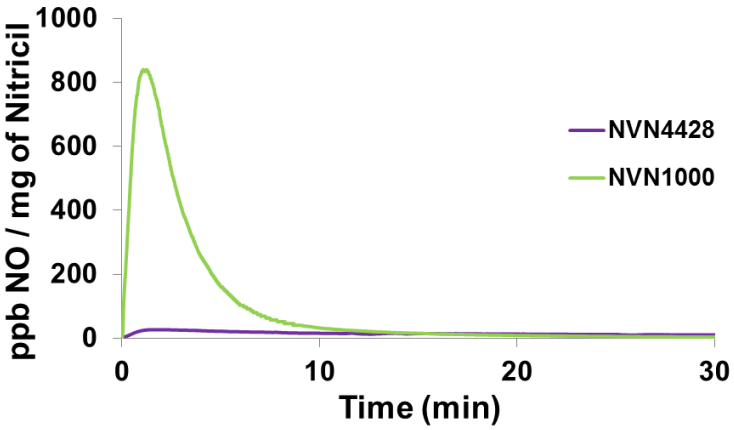
The full sequence of experiments against each pathogen demonstrate that different pharmacodynamic parameters, including the rate of nitric oxide release, influence antimicrobial efficacy. This effect is further illustrated in Figure 6, where the amount of nitric oxide released from NVN1000 required for a 4-log reduction in bacteria for each of the ESKAPE pathogens is shown. The same amount of nitric oxide reduces *E. faecium, A. baumannii, and P. aeruginosa* by greater than 4-logs after a 4-hour exposure (150 µg NO), while twice that amount is required for the same reduction in *K. pneumoniae* (300 µg NO). Substantially higher doses of nitric oxide are required to attain a 4-log reduction in *E. cloacae* (600 µg NO) and *S. aureus* (1200 µg NO).

Bacterial Resistance

In the arms race against bacteria, bacteria have undoubtedly outpaced drug development. Bacteria employ two different strategies to acquire resistance: 1) they can acquire new genetic material (DNA) from another source or 2) they can mutate their own DNA. Like human DNA, bacterial DNA is

Figure 5. Nitric Oxide Release Profiles for NVN1000 and NVN4428. *Macromolecules can be designed with different maximum instantaneous fluxes of nitric oxide* **(A)** *as well as different half-lives ($t_{1/2}$) and overall duration of release* **(B).**

A. Nitric Oxide Flux



B. Nitric Oxide Release

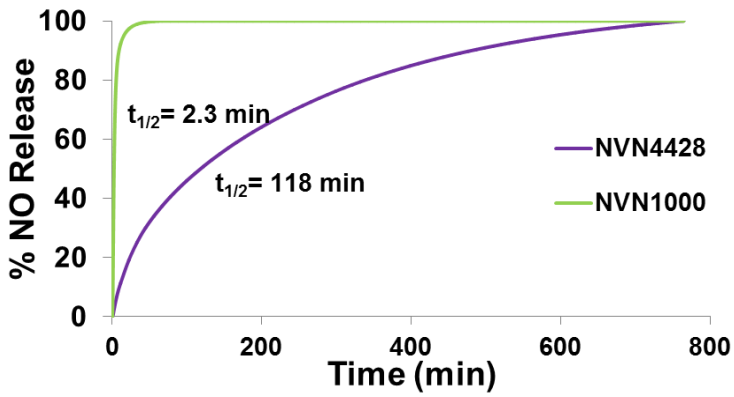
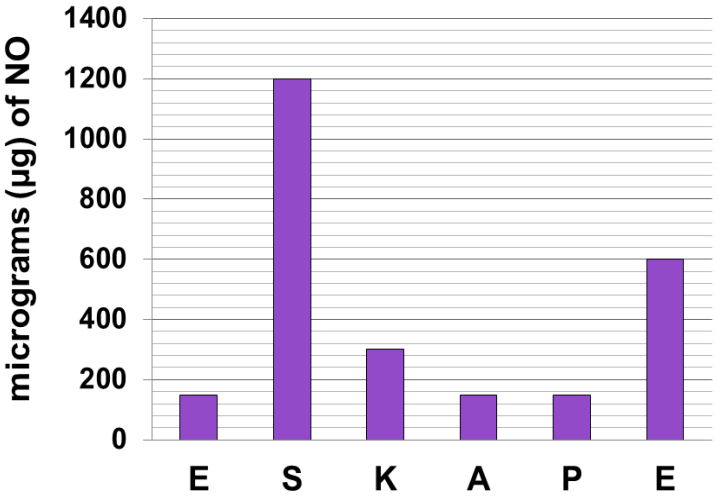


Figure 6. Amount of nitric oxide released from NVN1000 associated with a 4-log reduction of the ESKAPE pathogens *(listed in Figure 2).*



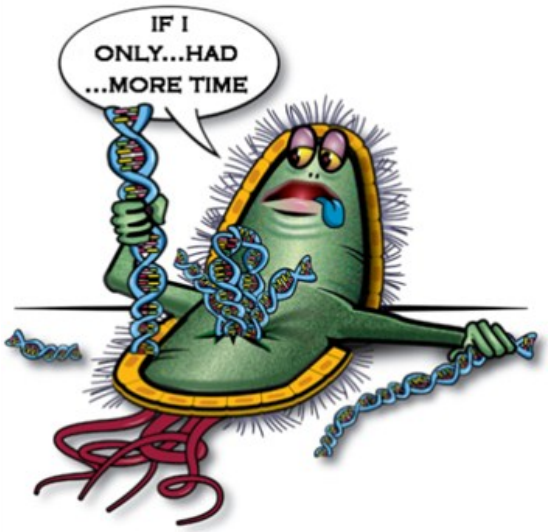
continually acquiring random mutations at a low basal rate. When a stressor is applied, like an antibiotic, the "selective pressure" kills or inhibits the growth of sensitive bacteria. Thus, the stressor "selects" bacteria with a beneficial mutation, promoting their survival and allowing them to grow more quickly and eventually predominate the bacterial population.

The ESKAPE pathogens *S. aureus* and *Enterococcus* are capable of acquiring resistance by exchanging genes through the acquisition of plasmids- circular pieces of DNA that are independent from bacterial chromosomal DNA. Isolates of *S. aureus* have already been identified that are fully resistant to vancomycin, the last resort in the treatment of staphylococcal infections. These isolates appear to have acquired the gene for vancomycin resistance *vanA* from *E. faecalis* (Arias 2012), a member of the normal intestinal flora, which can cause nosocomial infections in immunocompromised individuals.

Gram-negative bacteria possess intrinsic defense mechanisms that complicate effective treatment with antibiotics. While Gram-positive bacteria have a single cell wall which must be traversed by antimicrobials, Gram-negative bacteria possess an extra outer cell membrane which further impedes drug penetration. Furthermore, the Gram-negative pathogens have developed additional defense mechanisms to evade the effects of antibiotics including: the ability to activate efflux pumps allowing bacteria to actively pump antibiotics out of the cell; the expression of proteins which "chew-up", via enzymatic hydrolysis β-lactam antibiotics; and the ability to selectively shut down protein channels which have been exploited for the delivery of antibiotics into the bacterial cell. The result of these complications is the development of 3rd, 4th, and 5th generation antibiotics that impart incremental changes over existing therapies.

The numerous pathways resulting in bacterial cell damage upon exposure to nitric oxide account for its rapid bactericidal activity. As Stratton emphasized previously - **"Dead Bugs Don't Mutate."** Resistance is generated by exposure to bacteriostatic agents, or insufficient concentrations of bactericidal agents, which allow the survival of a few microorganisms. The survival of even a few bacteria retains the potential for development and propagation of resistance. As bactericidal agents are less likely to foster resistance than bacteriostatic agents, the general hypothesis is that nitric oxide-resistant strains of bacteria are unlikely to develop (Nathan 2000, Privett and Friedman 2011).

Figure 7. Dead bugs don't mutate. *Bactericidal agents eliminate the population before bacteria have the opportunity to mutate their DNA to generate resistance.*



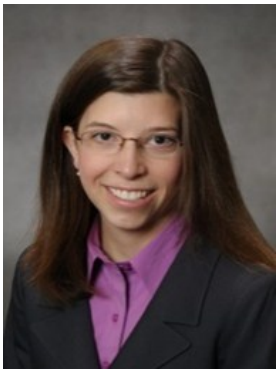
Bactericidal Mechanisms of Nitric Oxide

The time-kill study results demonstrate the bactericidal efficacy of nitric oxide. The antimicrobial activity of nitric oxide is due in large part to its reaction with oxygen species to form nitrosative (e.g. N_2O_3) and oxidative (e.g. OONO-) intermediates (Wink 2011). Nitrosative damage includes thiol nitrosation of membrane proteins and deamination of primary amines within DNA. The production of peroxynitrite results in oxidative DNA damage and membrane lipid peroxidation. Additional targets of these reactive species

include enzymes that contain essential redox centers (e.g., Fe-S clusters, heme, non-heme iron, copper, reactive thiols) (Fang 1997, De Groote 1995). Many of the enzymes within this category are required for essential microbial metabolic processes including amino acid synthesis, aerobic metabolism, and DNA replication (Richardson 2011, Hyduke 2007, Stevanin 2000, Gardner 1998).

By inhibiting multiple bacterial targets nitric oxide therapy represents a radical approach with a broad spectrum of activity and a low propensity for the development of bacterial resistance.

Novan Therapeutics is a development stage company subject to the risks and uncertainties associated with product development.



About the Author

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Dr. Coggan has extensive experience with the ESKAPE pathogen *Pseudomonas aeruginosa,* which was the focus of her dissertation research. She also spent significant time working with clinical isolates of *Staphylococcus aureus*. As a graduate student, Dr. Coggan was a fellow in the University of North Carolina at Chapel Hill's Translational Medicine program, which provides PhD students with a broad familiarity with human disease and the clinical perspective, including a working knowledge of human pathophysiology, the clinical presentation of diseases, the vocabulary of patient care, and the mechanics and ethical issues related to clinical investigation. Dr. Coggan is a member of the American Society of Microbiology.

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