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Nitric oxide-dependent killing of aerobic, anaerobic and persistent *Burkholderia pseudomallei*

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Abstract

Burkholderia pseudomallei infections are fastidious to treat with conventional antibiotic therapy, often involving a combination of drugs and long-term regimes. Bacterial genetic determinants contribute to the resistance of *B. pseudomallei* to many classes of antibiotics. In addition, anaerobiosis and hypoxia in abscesses typical of melioidosis select for persistent populations of *B. pseudomallei* refractory to a broad spectrum of antibacterials. We tested the susceptibility of *B. pseudomallei* to the drugs hydroxyurea, spermine NONOate and DETA NONOate that release nitric oxide (NO). Our investigations indicate that *B. pseudomallei* are killed by NO in a concentration and time-dependent fashion. The cytotoxicity of this diatomic radical against *B. pseudomallei* depends on both the culture medium and growth phase of the bacteria. Rapidly growing, but not stationary phase, *B. pseudomallei* are readily killed upon exposure to the NO donor spermine NONOate. NO also has excellent antimicrobial activity against anaerobic *B. pseudomallei*. In addition, persistent bacteria highly resistant to most conventional antibiotics are remarkably susceptible to NO. Sublethal concentrations of NO inhibited the enzymatic activity of [4Fe-4S]-cofactored aconitase of aerobic and anaerobic *B. pseudomallei*. The strong anti-*B. pseudomallei* activity of NO described herein merits further studies on the application of NO-based antibiotics for the treatment of melioidosis.

Keywords

antibiotics; antimicrobials; melioidosis; reactive nitrogen species; therapy; [4Fe-4S] clusters

Introduction

The Gram-negative, facultative intracellular pathogen *B. pseudomallei* is the causative agent of melioidosis. *B. pseudomallei* is a soil saprophyte widely distributed in Southeast Asia and Northern Australia. More recently, this microorganism has become an emerging opportunistic pathogen in Central and South America, and other tropical regions of the world (11). Humans and animals are often infected with *B. pseudomallei* through the

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respiratory tract upon contact with contaminated aerosols. Mammalian hosts can also be exposed to this microorganism through the oral mucosa after the ingestion of tainted food or water, or through skin abrasions and wounds (37).

The clinical presentations of *B. pseudomallei* infections in humans range from acute or chronic pneumonia to fatal septicemia. The progression of melioidosis is frequently associated with the development of pulmonary, hepatic and splenic abscesses that, for unclear reasons, involve prostatic tissue in Thai patients. Abscesses pose a physical barrier for the diffusion of antibiotics, and are consequently a major concern in the treatment of melioidosis (12). The extreme resistance of *B. pseudomallei* to multiple antibiotics further complicates antibiotic therapy of this fastidious infectious agent. The combination of microbial determinants and host physical barriers likely explain why lengthy regimes involving multiple classes of antibiotics are a common clinical practice in the therapy of melioidosis (7, 25). Despite extended treatments that last about 20 weeks, *B. pseudomallei* still relapse in 5-10% of the patients (8, 13, 26). Its intrinsic resistance to antibiotics, persistence in infected tissues, historical utilization as a bioweapon and the lack of a vaccine have led authorities in the United States to categorize *B. pseudomallei* as a category B select agent that requires BSL3 containment (9, 46, 50, 51).

The diatomic radical nitric oxide (NO) can be generated by NO synthase hemoproteins widely distributed among eukaryotes and present in some prokaryotic microorganisms. NO produced by the inducible NO synthase (iNOS) is a common component of the host arsenal against diverse pathogenic bacteria. For example, *B. mallei* and *Pseudomonas aeruginosa* are not only susceptible to iNOS-mediated cytotoxicity but they are also killed by a variety of reactive nitrogen species, including NO (4, 20, 21, 45, 53). The killing of these microorganisms by NO is quite remarkable, because most of the antimicrobial actions that this diatomic radical exerts against most microorganisms are manifested as cytostasis (14, 48). The iNOS hemoprotein appears, however, to be dispensable for host defense against *B. pseudomallei* in an experimental murine model of melioidosis (2, 3). The reason for the superfluous role of NO in the course of *B. pseudomallei* infection might reflect the capacity of this facultative intracellular pathogen to inhibit NO synthesis rather than the intrinsic resistance of these bacteria to reactive nitrogen species. Two mechanisms have been invoked by which *B. pseudomallei* minimize the production of NO by professional phagocytes. First, compared to lipopolysaccharide from enteric bacteria, the lipopolysaccharide of *B. pseudomallei* is hypostimulatory (1, 18). Second, although poorly characterized, *B. pseudomallei* downregulate NO synthesis by IFN γ -activated macrophages in an RpoS-dependent manner (45). The low levels of NO generated in response to *B. pseudomallei* infection probably confer a selective advantage to this microorganism. The concerted efforts of *B. pseudomallei* to inhibit NO synthesis may reflect hypersensitivity of this intracellular pathogen to NO oxidative and nitrosative congeners, offering unique opportunities for treating melioidosis. Given the NO-mediated killing of *B. mallei* and *P. aeruginosa*, we tested whether compounds that generate NO exert antimicrobial activity against *B. pseudomallei*. Our investigations indicate that several NO donors effectively kill aerobic, anaerobic and persistent populations of *B. pseudomallei*.

Materials and Methods

Bacterial strains and growth conditions

Strain K96243, a clinical isolate of *B. pseudomallei*, was grown in the BSL3 laboratory of the Department of Microbiology, University of Colorado School of Medicine, a facility certified by CDC for work with select agents. *B. mallei* strain ATCC 2344 and *B. thailandensis* E264 wild type strain were also used in the course of these investigations. The bacteria were grown overnight to stationary phase in Luria Bertani (LB) broth supplemented

with 4% (w/v) glycerol (LBG) in a shaker incubator at 37°C and 315 RPM (New Brunswick Innova, Edison, NJ). Overnight cultures were diluted 1:100 in LBG broth or E-salts minimal medium supplemented with either 0.4% (w/v) glucose and 1% (w/v) yeast extract (EGYE), or 0.1% (w/v) casamino acids (ECA). Log phase bacteria were collected for experimentation at an OD₆₀₀ of about 0.6. Bacterial cultures for anaerobic testing were pelleted by centrifugation and the samples moved into a Sheldon Bactron Anaerobic Chamber (Sheldon Manufacturing Inc., Cornelius, OR) containing a standard anaerobic gas mixture (5% H₂, 5% CO₂, 90% N₂). The *B. pseudomallei* bacterial pellets were resuspended in EGYE culture medium that had been deoxygenated in the anaerobic chamber for 24 h. The bacteria were adapted to the anaerobic environment for 2 h prior to challenge with the NO donors. Long-term anaerobiosis was achieved using shaking tubes in the “Wayne” model first described for work with *Mycobacterium tuberculosis*, and recently adapted for work with *B. pseudomallei* (16, 17). Hungate anaerobic glass tubes containing magnetic stir bars and filled with 14 ml of LBG broth were inoculated with log phase *B. pseudomallei* and sealed with Hungate stoppers and airtight screw caps. These cultures were stirred at 37°C for 31 days. Under these conditions, O₂ and nutrients are gradually depleted from the culture medium and the bacteria are subjected to prolonged anaerobiosis.

Killing Assays

Aerobic bacteria grown to an OD₆₀₀ of 0.6 were diluted 1:50 into the appropriate media and the samples aliquoted into round bottom 96-well plates (Evergreen Scientific, Los Angeles, CA). The bacterial cells were incubated with increasing concentrations of spermine NONOate (Cayman Chemical, Ann Arbor, MI) at 37°C for 2.5 h. For assays requiring anaerobic conditions, overnight cultures were diluted 1:50 into EGYE medium, and grown aerobically until they reached an OD₆₀₀ of 0.6. Bacterial pellets were moved into an anaerobic chamber where they were resuspended in EGYE medium that had been depleted of O₂ for 24 h. After 2 h of adaptation to the anaerobic environment, the bacteria were seeded into 96-well plates introduced into the anaerobic chamber 24 h earlier. The bacteria were then challenged with the NO donor spermine NONOate. Bacteria grown in the long-term “Wayne” anaerobic model of persistence were treated with the NO donor DETA NONOate (Cayman Chemical) during the last 24 h of culture. DETA NONOate and spermine NONOate were diluted to the desired concentrations in the indicated culture media from fresh 150 mM stocks prepared in 10 mM Tris-Cl buffer, pH 8.5. The half-lives of spermine NONOate and DETA NONOate at 37°C, pH 7.4, are 39 min and 20 h, respectively. At the indicated times, the surviving bacteria were diluted and plated on LB agar. The % of survival was calculated as (cfu t_n/cfu t₀) × 100.

Determination of MIC and MBC values

Standard minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) were estimated in LBG broth inoculated with 10⁶ CFU/ml of *B. pseudomallei*. Hydroxyurea (Sigma-Aldrich), spermine NONOate or DETA NONOate were tested at concentrations that ranged from 62.5 µg/ml to 1 mg/ml. The cultures were serially diluted and spotted on LB agar plates. The MIC was defined as the lowest concentration of the NO donor that visibly inhibited bacterial growth after 24 h of culture at 37°C with shaking, whereas the MBC was defined as the concentration of the NO donor that killed 99.9% of the original inoculum.

Aconitase enzymatic assay

Thirty-six ml of bacteria were grown in the presence of O₂ in EGYE or ECA media to an OD₆₀₀ of about 0.6. The bacterial cultures were treated with 100 µM spermine NONOate for 10 min. To study the effects of NO on the aconitase activity in the absence of O₂, bacterial pellets were introduced into the anaerobic chamber and the cells resuspended in

EGYE medium that had been deoxygenated in the anaerobic chamber for 24 h. After 2 h of adaptation in the anaerobic environment, the bacteria were challenged with 100 μ M spermine NONOate for 10 min. Under these conditions, the viability of the bacteria was unaffected. The anaerobic cultures were removed from the chamber and the rest of the manipulations were conducted aerobically. The bacteria from the anaerobic and aerobic cultures were pelleted by centrifugation at 4000 RPM for 5 min. The pellets were washed in 4.5 ml of 50 mM Tris-HCl buffer, pH 7.4, and the bacteria were lysed after the addition of 1 ml of B-PER buffer (Pierce, Rockford, IL) while vortexing for 30 sec. Two milliliters of 50 mM Tris-HCl buffer, pH 7.4, were added to the suspension and the lysates clarified by centrifugation for 1 min at 14,000 RPM. The clear lysates were filter-sterilized twice and the surface of the tubes decontaminated in preparation for their removal from the BSL-3 facility. The samples were snap-frozen in liquid nitrogen and stored in liquid nitrogen until the enzymatic activity was assessed. Protein concentrations in the samples were normalized and the presence of aconitase activity tested in 30-60 μ g protein as assessed by the formation of cis-aconitate in 50 mM Tris-HCl buffer, pH 7.4, containing 30 mM isocitrate. Aconitase activity is expressed as OD₂₄₀/50 μ g protein.

Results

Susceptibility of *Burkholderia* species to NO

We recently reported that *B. mallei* are highly susceptible to NO (21). In this study, we investigated the sensitivity of the related microorganisms *B. mallei*, *B. pseudomallei* and *B. thailandensis* to chemically generated NO. The NO donor spermine NONOate, which has a half-life of about 39 min and releases 2 moles of NO per mole of the compound, was used for most of these investigations. Bacteria grown to stationary phase were exposed to increasing concentrations of spermine NONOate (figure 1A). Stationary phase *B. mallei* subculture in fresh LB broth grew 3-fold during the 2.5 h of the experiment. As expected (21), *B. mallei* were highly susceptible to increasing concentrations of NO. The addition of 25 μ M spermine NONOate killed over 99.9% of *B. mallei* after 2.5 h of treatment. Stationary phase *B. pseudomallei* and *B. thailandensis* were more resistant to NO than *B. mallei*. We also tested the susceptibility of rapidly growing *Burkholderia* species to NO. As seen with stationary phase bacteria, log phase *B. mallei* were readily killed by as little as 25 μ M spermine NONOate (figure 1B). Although slightly less susceptible than *B. mallei*, log phase *B. pseudomallei* were also killed quite efficiently by spermine NONOate. In fact, 100 μ M spermine NONOate reduced the number of *B. pseudomallei* 10,000-fold. In contrast to *B. mallei* and *B. pseudomallei*, log phase *B. thailandensis* were relatively resistant to spermine NONOate, since only about 10% of the population was killed by 100 μ M of the NO donor. The susceptibility of log phase bacteria to NO was studied in more detail in time kill curves (figure 1C). The addition of 100 μ M spermine did not exert any measurable anti-*Burkholderia* activity 2h after exposure. In contrast, killing of log phase *B. pseudomallei* by 100 μ M spermine NONOate was linear for about 2 h, a time when approximately 99.9% of the original inoculum succumbed to the treatment. After 2 h, however, the surviving bacteria appear to be resistant to NO. These data suggest that the survivors express either innate or adaptive resistance to this diatomic radical. Alternatively, the survivors may represent persister cells in the population of *B. pseudomallei*. The anti-*B. pseudomallei* activity of spermine NONOate is dependent on the NO generating capacity of the drug, since the spermine control exerted no measurable antibiotic activity (not shown). Moreover, NO₂⁻ and NO₃⁻, terminal oxidative products of NO, did not exert any appreciable killing of *B. pseudomallei* (not shown). Although *B. mallei* were slightly more susceptible to the cytotoxicity of NO, the time kill curves of *B. mallei* and *B. pseudomallei* followed similar kinetics. Early on, *B. thailandensis* were also efficiently killed by spermine NONOate; however, this microorganism resumed growth 1.5 h after challenge. The surviving bacteria

after 2.5 h of 100 μ M spermine NONOate treatment could represent a persistent population or a mutant population selected after NO treatment. While the former should become susceptible to NO after culture in fresh medium, the latter should remain resistant. To distinguish between these two possibilities, *B. pseudomallei* that survived challenge with 100 μ M spermine NONOate were grown overnight to stationary phase in LB broth. Log phase bacteria grown from NO-treated controls were readily killed upon exposure to 100 μ M spermine NONOate (figure 1D). These findings suggest that the fraction of bacteria that survive 2.5 h after NO challenge are a persistent population genetically susceptible to NO but in a physiological state refractory to NO cytotoxicity.

Killing of *B. pseudomallei* by antibiotics that generate NO

In the following experiments, we determined the MIC and MBC values for 3 different NO donors (table 1). *B. pseudomallei* grown to an OD₆₀₀ of 0.2 were seeded in 10 ml of LBG broth at a concentration of 10⁶ CFU/ml. Increasing concentrations of the NO donors were added to the flasks and the MIC values recorded 24 h after challenge. Hydroxyurea showed the lowest MIC (i.e., 125 μ g/ml) followed by DETA NONOate and spermine NONOate (i.e., 250 μ g/ml and >1 mg/ml, respectively). The anti-*B. pseudomallei* cytotoxicity of DETA NONOate appears to be associated with the NO generated by this drug, because the addition of 250 μ g/ml polyamine DETA to LB broth did not inhibit bacterial growth after overnight culture. We calculated that 250 μ g/ml of DETA NONOate generates a steady concentration of 1.5 μ M NO in the media for the duration of the experiment. On the other hand, our estimates indicate that 1 mg/ml spermine NONOate generates 3.8 mM NO during the first hour, ever declining to about 1.5 μ M and 90 nM after 7 h and 10 h, respectively. The MBC values for all the NO donors tested were higher than 1mg/ml. Further analysis indicated that 250 μ g/ml of DETA NONOate did not reduce the viability of *B. pseudomallei* 2.5 h after challenge, whereas 1 mg/ml spermine NONOate killed about 99% of the bacterial cells (figure 2). Neither 250 μ g/ml DETA nor 1 mg/ml spermine controls had any effects on bacterial viability 2.5 h after challenge. These data indicate that the anti-*B. pseudomallei* activity of DETA NONOate is likely due to the cytostasis exerted by the sustained fluxes of NO over an extended period of time. Despite the initial onslaught, the few survivors likely overcome the diminishing fluxes of NO generated by spermine NONOate.

Influence of culture media on the susceptibility of *B. pseudomallei* to NO

B. pseudomallei tripled in numbers 2 h after culture, irrespectively of the media used in these experiments (figure 3A). As seen in figure 1, *B. pseudomallei* grown to log phase in EGYE medium were highly susceptible to increasing concentrations of NO (figure 3A). The NO-dependent killing of *B. pseudomallei* seen in EGYE medium depended on the presence of yeast extract but not glucose. Titration experiments revealed that supplementation of E salts with 0.1% (w/v) yeast extract supported the anti-*B. pseudomallei* activity of NO. In contrast, *B. pseudomallei* grown in E salts medium supplemented with 0.4% (w/v) glucose and/or 0.1% (w/v) casamino acids were relatively resistant to the cytotoxicity of spermine NONOate (figure 3). We considered whether the killing of *B. pseudomallei* by NO depended on vitamins and salts present in yeast extract. The addition of biotin, nicotinic acid, pyridoxin, riboflavin, thiamine, vitamin B, ZnCl₂, CaCl₂ or CuSO₄ to ECA medium did not, however, improve the killing of *B. pseudomallei* by spermine NONOate (not shown). These findings suggest that the cytotoxicity of NO against this opportunistic pathogen is best seen in complex medium. Accordingly, spermine NONOate exerted excellent anti-*B. pseudomallei* activity against bacteria grown in LB broth, especially if 4% glycerol (v/v) was present in the medium (figure 3B). Our investigations suggest that the metabolic state of *B. pseudomallei* affects the susceptibility of this opportunistic pathogen to NO.

Influence of anaerobiosis on the susceptibility of *B. pseudomallei* to NO

B. pseudomallei infections are quite resistant to several classes of antibiotics. Classically, the antibiotic resistance of *B. pseudomallei* has been associated with a variety of genetic or physiologic bacterial determinants (6, 23, 27, 32, 33, 41, 44, 49). Recently, we reported that anaerobiosis induces tolerance of *B. pseudomallei* to antibiotics frequently used for treating melioidosis (16). Since *B. pseudomallei* likely experience anaerobiosis or severe hypoxia in several cellular and anatomical compartments during the course of the infection, the antimicrobial activity of NO against anaerobic bacteria was tested. Figure 4A shows that *B. pseudomallei* were readily killed by increasing concentrations of spermine NONOate when the cultures had been pre-adapted for 2 h in the anaerobic environment. In fact, over 99.99% of the bacteria were killed by 1 h of exposure to 400 μ M spermine NONOate (figure 4B). The number of bacteria did not change appreciably thereafter. The genome of *B. pseudomallei* encodes several nitrate reductases for growth in the absence of O₂ using NO₃⁻ as a terminal electron acceptor. Neither the addition of NO₃⁻ nor NO₂⁻ to EGYE medium changed the excellent NO-dependent killing of anaerobic *B. pseudomallei* (not shown).

B. pseudomallei were gradually adapted to anaerobiosis in anaerobic Hungate tubes whereby the bacteria consume all available oxygen within 24 h at which point they survive under anaerobic conditions for several days (16). After several days under anaerobic conditions, most bacteria die leaving 0.1% of the anaerobic population that is stable and capable of persisting up to at least one year in a non-replicating state. At day 30 in the Hungate model, the persister population is tolerant to antibiotics effective against both aerobic as well as anaerobic *B. pseudomallei* (16). On day 30 of the Hungate model, the slow NO releaser DETA NONOate was inoculated into the cultures and the surviving bacteria recorded 24 h later. This method of adaptation to anaerobic conditions yielded bacteria highly susceptible to NO (figure 4C). In fact, no viable bacteria were recovered after overnight challenge with 2 mM DETA NONOate, a condition that steadily maintains about 2 μ M NO in the culture medium. Thus, this persister population, which is entirely tolerant to conventional antibacterial treatment, appears to be highly susceptible to NO.

NO readily inactivates *B. pseudomallei* aconitase enzymatic activity

The anti-*B. mallei* activity of NO is associated with the inactivation of metalloproteins bearing [4Fe-4S] clusters (21). We therefore tested whether sublethal concentrations of spermine NONOate had any effect on the enzymatic activity of the [4Fe-4S]-cofactored aconitase. *B. pseudomallei* grown aerobically in EGYE medium to an OD₆₀₀ of 0.6 had excellent aconitase activity (figure 5A). Having established the presence of aconitase activity in these cultures, we set out to determine whether NO had any effect on the enzymatic activity of this metalloprotein. To eliminate possible interpretation errors, we initially determined concentrations of spermine NONOate that did not affect bacterial viability under the culture conditions used for the aconitase assays. Screening of different NO concentrations showed that treatment of log phase *B. pseudomallei* with 100 μ M spermine NONOate for 10 min dramatically decreased aconitase enzymatic activity (figure 5A). The aconitase activity of cell extracts recovered from NO-treated *B. pseudomallei* while growing in EGYE medium was similar to untreated controls grown in ECA medium (figure 5A). Interestingly, 100 μ M spermine NONOate did not appear to affect the aconitase enzymatic activity of *B. pseudomallei* grown in ECA medium.

There is some controversy regarding the identity of the reactive nitrogen species responsible for the NO-dependent inactivation of aconitase. Peroxynitrite (ONOO⁻), an product of the reaction of NO with O₂⁻, readily oxidizes the Fe_a of the [4Fe-4S] cluster of aconitase, thereby inactivating the enzyme (5, 19). Some investigators, however, have contended that NO itself can inactivate aconitases through the nitrosylation of the solvent-exposed, electron

withdrawing Fe_q atom (15). Given the potent antimicrobial activity of NO against anaerobic *B. pseudomallei* (a condition that precludes ONOO⁻ formation), aconitase activity was examined in *B. pseudomallei* exposed to NO in the absence of O₂. As described above, *B. pseudomallei* grown to an OD₆₀₀ of 0.6 in EGYE medium and adapted for 2 h in an anaerobic chamber showed excellent aconitase enzymatic activity (figure 5B). Exposure of anaerobic *B. pseudomallei* to 100 μM spermine NONOate readily inhibited the aconitase enzymatic activity in these cultures. Remarkably, the aconitase activity of *B. pseudomallei* grown in an anaerobic environment appears to be even more susceptible to NO than controls grown aerobically. Cell lysates prepared, treated with NO and loaded into anaerobic cuvettes in the anaerobic chamber suffered a similar inhibition of aconitase activity to the one reported herein for NO-treated, live cultures (not shown).

Discussion

NO arrests the replication of phylogenetically diverse microorganisms such as *Candida albicans*, *E. coli* and *Salmonella enterica* (14, 38, 48). In contrast to these pathogens, *B. pseudomallei* belong to a select group of pathogenic bacteria, including *B. mallei*, *B. cepacia*, and *P. aeruginosa*, that are killed by NO (work herein and references 21, 52). *B. pseudomallei* appear to be more susceptible to NO than *P. aeruginosa*. Accordingly, reactive nitrogen species reduced the viability of *B. pseudomallei* about 10,000-fold, whereas these reactive species dropped the fraction of planktonic and mucoid *P. aeruginosa* about 10- to 100-fold (29, 31, 52). Consequently, as it has been proposed for *P. aeruginosa* (52), it might be worth evaluating the potential of NO-based antibiotics as therapeutic agents against melioidosis. NO was found to be quite efficient at killing aerobic and anaerobic *B. pseudomallei*. However, higher amounts of NO appear to be needed to kill the latter. This indicates that, even though NO kills *B. pseudomallei*, the presence of oxygen enhances the anti-*Burkholderia* activity of this diatomic radical.

The MIC and MBC values of the NONOates used in our studies define NO as a bacteriostatic drug against log phase *B. pseudomallei*. This seems surprising given the massive decline in bacterial viability that follows NO treatment. The expansion of a small population of survivors highly resistant to NO could explain the MIC and MBC values reported herein for spermine NONOate. NO does not appear to induce adaptive mutations in *Burkholderia*'s genome, since expansion of the resistant population in fresh LB broth yields highly susceptible bacteria. The fraction of bacteria resistant to nitrosative stress most likely represent a persistent population in a physiological state refractory to the cytotoxicity of NO. The two NO donors tested exhibited different MIC values against *B. pseudomallei*, likely reflecting an inverse correlation with the half-lives of these compounds. Under the experimental conditions tested, DETA NONOate steadily releases about 2 μM NO in the media. The low NO fluxes generated by DETA NONOate inhibit *B. pseudomallei* growth for extended periods of time. In comparison, spermine NONOate gives rise to a 3 mM NO bolus shortly after challenge, gradually diminishing to low nanomolar concentrations 10 h later. Given the extreme susceptibility of *B. pseudomallei* to this diatomic radical, future efforts to develop NO-based antibacterials against *B. pseudomallei* should favor drugs that allow for long-lasting release of small NO fluxes rather than chemicals that provide a short burst of NO. Persistent *B. pseudomallei* were even more susceptible to NO than log phase bacteria. The high susceptibility of persistent *B. pseudomallei* to NO is quite remarkable, because this population is tolerant to conventional antibiotics used against aerobic and anaerobic bacteria (16).

Treatment of infectious diseases with prodrugs that release NO is an attractive alternative to more classical NO donors. For example, the antibiotic nitroimidazole kills anaerobic *M. tuberculosis* through the formation of NO (42). Similarly, NO generated from the nitro

group of metronidazole is responsible for the compound's antimicrobial activity (30). Of relevance to our investigations, we recently reported excellent antimicrobial activity of metronidazole against anaerobic *B. pseudomallei* (16). H₂O₂, transition metals or hemoproteins bioactivate the production of NO from the prodrug hydroxyurea (24, 35). This antibiotic has been used in the clinic against human immunodeficiency virus, hepatitis C virus and Epstein-Barr virus (28, 34, 36). NO-mediated inactivation of ribonucleotide reductase and the consequent inhibition of deoxyribonucleotide biosynthesis are central to the mechanism of action of hydroxyurea. Interestingly, hydroxyurea exhibited the most profound anti-*Burkholderia* activity of all the NO-based antibiotics tested in the course of our studies. The widespread occurrence of anaerobic abscesses in *B. pseudomallei*-infected individuals warrants future evaluation of the therapeutic value of NO-dependent prodrugs in the treatment of these fastidious infections.

B. pseudomallei infections are remarkably fastidious to treat, often requiring long regimes and combined therapies. The expression of a variety of bacterial determinants is central to the antibiotic resistance of this opportunistic pathogen (23, 27, 32, 33, 41, 44, 49). Although much less studied, the complex lifecycle of *B. pseudomallei* within vertebrate hosts may also contribute to the lengthy regimes and frequent failure of the current therapy against melioidosis. Signals encountered during the course of the infection may select for persistent populations refractory to antibiotics that may otherwise be excellent choices when tested *in vitro*. For example, conditions mimicking the anaerobic environment of abscesses tolerate *B. pseudomallei* to a variety of antibiotics used in the clinic (16). Interestingly, our investigations show that persistent populations of *B. pseudomallei* selected in long-term anaerobic cultures become exquisitely sensitive to the antimicrobial actions of NO.

An alternative to NO-based antibiotics is to engage host signaling pathways for the induction of endogenous NO synthesis. There are obvious advantages to this immunostimulatory approach. 1) The enzymatic production of NO allows for the sustained release of this antimicrobial overtime. The superb anti-*B. pseudomallei* activity of slow NO releasing drugs highlights the importance of the latter concept. 2) Enzymatic production delivers NO to the site of the infection, thereby increasing local concentrations of the antimicrobial. And 3) the enzymatic production of NO takes place as long as the pathogen is present in the infected tissue, but ceases with its eradication. The localized and transient enzymatic production of NO have the added advantage of minimizing the collateral damage associated with the reaction of this diatomic radical with host cell biomolecules. The immunostimulatory approach has already been used in treating some of the syndromes associated with acute *B. pseudomallei* infection. It remains to be investigated whether and to what extent granulocyte colony stimulating factor adjuvant therapy used in septic complications of melioidosis works through the production of NO (10, 22, 40).

B. mallei, *B. pseudomallei*, and *B. thailandensis* showed drastically different susceptibilities to reactive nitrogen species. For example, log and stationary phase *B. mallei* were readily killed by NO, whereas *B. pseudomallei* were preferentially killed when treated during the exponential phase of growth. The nonpathogenic, environmental microorganism *B. thailandensis* is as susceptible as *B. pseudomallei* to the early antimicrobial activity of NO. However, *B. thailandensis* resume growth shortly after exposure to the radical. The growth of *B. thailandensis* in the presence of NO resembles the gain in cell mass of *Staphylococcus aureus* following the utilization of NO-resistant metabolic pathways (39). The idea that metabolism is an important target of the antibacterial activity of NO is supported by the fact that the type of media greatly influenced the degree of anti-*Burkholderia* activity of NO. The expression of sensitive molecular targets could explain the high susceptibility of *B. pseudomallei* grown in EGYE medium to nitrosative stress. Accordingly, the composition of the media affected the differential susceptibility of aconitase activity of *B. pseudomallei*

exposed to NO. In addition, selective expression of antinitrosative defenses such as the flavohemoprotein may have contributed to the differential susceptibility of log and stationary phase bacteria to NO-mediated killing.

Metalloproteins bearing [4Fe-4S] clusters such as aconitases are important targets of the NO-mediated cytotoxicity against *B. mallei* (21). Our current investigations indicate that the aconitase activity of *B. pseudomallei* is also exquisitely sensitive to the antimicrobial actions of NO. The NO-mediated inhibition of aconitase is more effective in anaerobic than aerobic bacteria. This observation is remarkable because NO itself, and not ONOO⁻ arising from the interaction of NO and O₂⁻, appears to be sufficient for the inhibition of aconitase enzymatic activity described herein. The mechanism of inhibition likely involves a rapid reversible phase followed by a long-lasting irreversible inactivation of the iron-nitrosyl [4Fe-4S] cluster (15, 43). The *B. pseudomallei* genome encodes two A-type aconitases, a class of aconitase that is resistant to oxidative stress (47). As discussed above, the aconitase activity of *B. pseudomallei* grown in EGYE medium, but not in ECA medium, was inactivated upon exposure to NO. These findings raise the possibility that the two A-type aconitases of *B. pseudomallei* are not only differentially expressed at different O₂ tensions and in response to different carbon sources, but may also exhibit different reactivity with nitrosative and oxidative congeners of NO. These interesting possibilities await further investigation. *B. pseudomallei* aconitase activity was inhibited 10 min after exposure to NO, while a noticeable decline in viability was recorded 20 min later. In contrast to cell death associated with damage to the cell membrane, the NO-mediated inactivation of central metabolic pathways probably explains the slow onset of killing. The anti-*B. pseudomallei* activity of NO likely represents its damaging effect on multiple biomolecules, including aconitase. Enzymes with [4Fe-4S] clusters such as phosphogluconate dehydratase of the Entner-Doudoroff pathway could be inactivated upon exposure to NO. In addition, the oxidation of redox active thiols in enzymes such as glyceraldehyde-3-phosphate dehydrogenase of glycolysis or the inactivation of terminal cytochromes of the electron transport chain could contribute to the NO-dependent cytotoxicity against *B. pseudomallei*. Long-term nitrosylation of metal prosthetic centers of the electron transport chain may be of consequence to this strict aerobe. Metalloproteins containing zinc fingers and metal cofactors, and DNA molecules are also likely targets of the anti-*B. pseudomallei* activity of NO.

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Highlights

Burkholderia pseudomallei infections are fastidious to treat with conventional antibiotic therapy

B. pseudomallei are extraordinarily susceptible to a variety of NO donors

NO kills stationary and log phase, anaerobic and persistent *B. pseudomallei*

NO could be considered as potential treatment to treat melioidosis caused by *B. pseudomallei* infections

Figure 1. Cytotoxicity of NO against stationary and log phase *Burkholderia* species

Stationary (A) and log (B) phase *B. pseudomallei*, *B. thailandensis*, and *B. mallei* were treated in EGYE (0.4% glucose, 1% yeast extract E salts medium) medium with increasing concentrations of the NO donor spermine NONOate. The fraction of bacteria surviving in the cultures was estimated 2.5 h after the addition of the NO donor. The survival of log phase bacteria was followed overtime after treatment with 100 μM spermine NONOate (sNO) (C). When expanded after overnight culture in LB broth, the apparently persistent population of *B. pseudomallei* (Bps) became susceptible to 100 μM sNO when grown in LB broth to log phase (log pers) (D). The data are the mean ± SD from 8-12 individual observations from 3 separate experiments.

Figure 2. Comparison of the anti-*B. pseudomallei* activity of spermine NONOate and DETA NONOate

The bacteria were exposed to 250 $\mu\text{g/ml}$ DETA, 250 $\mu\text{g/ml}$ DETA NONOate, 1 mg/ml spermine, or 1 mg/ml spermine NONOate as described for the experiments in table 1. Control represents the polyamine parent compound, whereas NONOate is the NO releasing derivative. The fraction of surviving bacteria was recorded 2.5 h after challenge. The data are the mean $\pm$ SD from 8 individual observations from 2 separate experiments.

Figure 3. Influence of growth media in the susceptibility of exponential phase *B. pseudomallei* to NO

(A) *B. pseudomallei* were grown aerobically to log phase in EGYE (0.4% glucose, 1% yeast extract E salts medium), ECA (0.4% casamino acids E salts medium) or EGCA (0.4% glucose, 0.4% casamino acids E salts medium). Bacterial cells grown to OD₆₀₀ of 0.6 were challenged with increasing concentrations of the NO donor spermine NONOate. The fraction of bacteria remaining in the wells was calculated 2.5 h after NO treatment. For the experiments in panel B, the bacteria were grown in LB broth or LBG (LB broth supplemented with 4% glycerol). The data are the mean $\pm$ SD from 4-12 individual observations from 2 separate experiments.

Figure 4. Susceptibility of anaerobic *B. pseudomallei* to chemically generated NO

The bacteria were grown to the exponential phase in EGYE medium and adapted for 2 h in an anaerobic chamber. The bacterial survival was scored 2.5 h after the addition of increasing concentrations of spermine NONOate (A) or after the indicated times of exposure to 400 μM spermine NONOate (sNO) (B). The data are the mean $\pm$ SD from 5 individual observations from 2 separate experiments. For panel C, *B. pseudomallei* were grown in a modified “Wayne” model of anaerobiosis. Bacteria grown overnight in LB broth were subcultured 1:200 in LB broth to an OD_{600} of 0.6. Log phase bacteria were stirred in Hungate tubes for 31 days. The NO generator DETA NONOate was inoculated into the cultures during the last day of culture. The data the mean $\pm$ SD from 6 observations collected in 2 separate days. Non viable cultures (n.v.c.).

Figure 5. Effects of NO on the aconitase activity of aerobic and anaerobic *B. pseudomallei*
 Aconitase enzymatic activity was determined in log phase bacteria grown to OD₆₀₀ 0.6 in EGYE or ECA media (A). Selected bacterial cultures were treated with 100 μ M spermine NONOate (sNO) for 10 min. The effect that sNO has on the aconitase activity of anaerobic *B. pseudomallei* grown in EGYE medium is shown in B. The data are representative of 3 experiments performed on independent days.

Table 1Susceptibility of *B. pseudomallei* to NO-dependent antibiotics

	MIC ¹ (µg/ml)	MBC ² (µg/ml)
Hydroxyurea	125	>1000
Spermine NONOate	>1000	>1000
DETA NONOate	250	>1000

The data are from 3 independent experiments.

¹Minimal inhibitory concentration

²Minimal bactericidal concentration