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Activity of Norspermidine on Bacterial Biofilms of Multidrug-Resistant Clinical Isolates Associated with Persistent Extremity Wound Infections

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Abstract

Biofilm formation is a major virulence factor for numerous pathogenic bacteria and is cited as a central event in the pathogenesis of chronic human infections, which is in large part due to excessive extracellular matrix secretion and metabolic changes that occur within the biofilm rendering them highly tolerant to antimicrobial treatments. Polyamines, including norspermidine, play central roles in bacterial biofilm development, but have also recently been shown to inhibit biofilm formation in select strains of various pathogenic bacteria. The aim of this study was to evaluate *in vitro* the biofilm dispersive and inhibitory activities of norspermidine against multidrug-resistant clinical isolates of *Acinetobacter baumannii* (n=4), *Klebsiella pneumoniae* (n=3), *Pseudomonas aeruginosa* (n=5) and *Staphylococcus aureus* (n=4) associated with chronic

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AUTHORS' CONTRIBUTIONS

APC, RLW and CJS conceived and designed the experiments. RLW, SCB, RAG and CJS performed the experiments. APC and CJS analyzed the data. APC, KM, KSA, JCW and CJS contributed to preparation and review of the final manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests

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extremity wound infections using the semi-quantitative 96-well plate method and confocal laser microscopy. In addition to the antibiofilm activity, biocompatibility of norspermidine was also evaluated by measuring toxicity *in vitro* to human cell lines and whole porcine tissue explants using MTT viability assay and histological analysis. Norspermidine (5 to 20 mM) had variable dispersive and inhibitory activity on biofilms which was dependent on both the strain and species. Of the clinical bacterial species evaluated herein, *A. baumannii* isolates were the most sensitive to the effect of norspermidine, which was in part due to the inhibitory effects of norspermidine on bacterial motility and expression of genes involved in the production of homoserine lactones and quorum sensing molecules both essential for biofilm formation. Importantly, exposure of cell lines and whole tissues to norspermidine for prolonged periods of time (> 24h) was observed to reduce viability and alter tissue histology in a time and concentration dependent manner, with 20 mM exposure having the greatest negative effects on both tissues and individual cell lines. Collectively our findings demonstrate that, similar to other polyamines, norspermidine displays both inhibitory and dispersive activities on biofilms of clinical multidrug-resistant bacterial isolates, in particular for strains of *A. baumannii*. Additionally our findings suggest that direct application may be considered on tissues, albeit for limited exposure times.

Keywords

norspermidine; polyamine; biofilm dispersal; biofilm inhibition; wound infection

INTRODUCTION

Bacteria can exist in either a planktonic, free-living individual state, or as a part of a surface-attached multicellular community embedded within a self-produced extracellular polymeric matrix, known as a biofilm (Costerton 1999; Costerton et al. 1999; Hall-Stoodley et al. 2004). To date numerous studies have demonstrated that bacterial biofilms can develop within host tissues and are cited as a significant bacterial virulence factor contributing to chronic wound infections (Bjarnsholt et al. 2008; Costerton et al. 1999; Hall-Stoodley et al. 2004; James et al. 2008). In contrast to planktonic bacteria, the excessive extracellular matrix secretion and concurrent metabolic changes that bacteria undergo while in a biofilm render them highly tolerant to antimicrobial treatments, often up to 1000-fold compared to their planktonic counterparts (Anderson and O'Toole 2008; Mah and O'Toole 2001; Koudhi et al. 2015). Consequently, the use of conventional antimicrobials for the treatment of biofilm-related infections, including chronic wounds, has limited efficacy as they fail to eradicate bacteria within the biofilms. Given the limitations of antimicrobials against biofilm bacteria, efforts by many groups have been directed towards identifying substances as well as developing strategies to inhibit and/or trigger dispersal of biofilms (Romling and Balsalobre 2012).

Biofilm development involves an elaborate life cycle, initiated by the attachment of microorganisms to the surface, followed by continued growth and eventual maturation. As the biofilm matures, resources become limited and waste products accumulate, initiating a cascade of events that lead to the disassembly of extracellular matrix and dispersal of bacteria from the biofilm. Studies evaluating the signals and cues utilized by bacteria to

initiate dispersal have led to the identification of a diverse group of secreted diffusible molecules including polyamines (Kaplan 2010; Kostakioti et al. 2013). Polyamines are small molecular weight, cationic molecules that have been broadly implicated in bacterial growth due to their ability to interact with nucleic acids and protein translation machinery, to exert antibacterial action against a number of various human pathogens, and contribute to disease pathogenesis by enhancing the expression of bacterial virulence factors (Karatan and Michael 2013). Polyamines have also been shown to play an essential role in biofilm formation (Wortham et al. 2007; Kolodkin-Gal et al. 2012). For example, putrescine is essential for biofilm formation in *Yersinia pestis* and *Escherichia coli* (Patel et al. 2006; Wortham et al. 2010). Similarly, spermidine and its shorter structural analog, norspermidine, have been reported to enhance biofilm formation in *Bacillus subtilis* and *Vibrio cholera* (Lee et al. 2009) respectively. Interestingly, polyamines have also been reported to inhibit biofilm formation of *B. subtilis*, *Staphylococcus aureus* and *E. coli*, and promote the disassembly of *B. subtilis* biofilms (Kolodkin-Gal et al. 2012; Karatan and Michael 2013). Based on the chemical nature of polyamines and the likely interaction with polyanions, interactions between polyamines with exopolysaccharides within the biofilm matrix were presumed to destabilize the matrix promoting disassembly and dispersal of biofilms (Kolodkin-Gal et al. 2012; Wortham et al. 2007). However, recent reports have proposed that norspermidine may inhibit biofilm formation through polysaccharide-independent mechanism of bacterial growth inhibition, in part explaining the inhibitory activity on biofilms of *B. subtilis* (Hobley et al. 2014). While these studies have provided preliminary evidence supporting the antibiofilm activities of norspermidine *in vitro*, these original studies were limited to only a few bacterial species and did not evaluate the biocompatibility of norspermidine, which is of particular importance given its suggested clinical use as a topical agent within studies. Therefore, the aim of the present study was to evaluate the inhibitory and dispersive activities of norspermidine on biofilms of several clinical isolates of multidrug-resistant organisms (MDRO) associated with chronic wound infections, as well as the potential clinical use as a topical agent by assessing biocompatibility using cell lines and whole tissues *in vitro*.

RESULTS

Preliminary screening of the inhibitory and dispersive activity of norspermidine against bacterial biofilms

Exposure of preformed biofilms to norspermidine, at concentrations between 0.1 – 20 mM, had only modest dispersive activity against preformed biofilms of representative strains of *K. pneumoniae* and *S. aureus*, reducing biomass between 20-38% of the untreated control group (Fig 1A). In contrast, significant dispersal activity of norspermidine was observed against preformed biofilms of isolates of *A. baumannii* and *P. aeruginosa* following overnight exposure to norspermidine. For *A. baumannii* and *P. aeruginosa*, dispersive activity was concentration-dependent, with significant activity at 20 mM, and between 5 and 20 mM, against *A. baumannii* and *P. aeruginosa* biofilms, respectively. In contrast to the dispersive activity against pre-formed biofilms, norspermidine had a much greater effect on inhibiting biofilm formation. The activity, as with dispersal, was also dose-dependent and most effective at concentrations between 5 and 20 mM. Notably, while the dispersive

activity of norspermidine was observed primarily against *A. baumannii* and *P. aeruginosa*, significant inhibitory activity was observed against the majority of the representative clinical isolates tested (Fig 1B). Importantly, while norspermidine was observed to have dispersive and inhibitory activity herein, this activity was only observed at much higher concentrations compared to other classes of polyamines previously shown to have antibiofilm activity, including putrescine and spermidine (Goytia et al. 2013; Nesse et al. 2015; Ramon-Perez et al. 2014) (Fig 1A-B).

Effect of norspermidine on bacterial growth

In light of previous reports suggesting that the antibiofilm activity of norspermidine may occur as a consequence of inhibition of bacterial growth, we also evaluated the effect of exposure to norspermidine on bacterial growth using the representative isolates evaluated above. With the exception of *S. aureus*, exposure to concentrations of norspermidine up to 20 mM had minor, albeit insignificant, activity on planktonic bacterial growth (Figure 1C). Increasing concentrations of norspermidine had the least effect on the growth of *P. aeruginosa*, whereas the 20 mM concentration resulted in only minor and insignificant impairments on the growth of *A. baumannii* and *K. pneumoniae*. In contrast to most of the other bacterial species evaluated, exposure of *S. aureus* to norspermidine at 20 mM essentially abolished the growth of the strain. Consistent with the growth curves, enumeration of each bacterial strain at similar time points at each tested concentration in a planktonic growth assay confirmed only moderate reductions of bacterial numbers in the presence of norspermidine up to 20 mM. *S. aureus* viability was significantly reduced following exposure to norspermidine at 20 mM. These findings suggest that the inhibitory and dispersive activity of norspermidine is, with the exception of *S. aureus*, largely independent of planktonic growth inhibition, and is exclusive to the biofilm phenotype.

Norspermidine inhibits and disperses biofilms of clinical isolates associated with chronic human infections

Consistent with preliminary screening results above, norspermidine at 5 and 20 mM demonstrated both dispersive and inhibitory activity against a panel of genetically diverse clinical isolates associated with chronic human infections (Figure 2A-B). The activities of norspermidine against the biofilms of clinical isolates were both species- and strain-dependent. Notably, clinical isolates of *A. baumannii* were the most sensitive to the effects of norspermidine. Although the activity was variable amongst the strains, exposure to norspermidine on average resulted in 60% dispersion and 80% inhibition of *A. baumannii* biomass. In contrast, the dispersive activity of norspermidine was variable against isolates of *P. aeruginosa* and *S. aureus*. For isolates of *K. pneumoniae*, dispersive activity tended to increase with rising norspermidine concentration, with the greatest effect on biofilm inhibition following exposure to 20 mM. Importantly, for *P. aeruginosa*, exposure to norspermidine in some cases significantly enhanced biofilm formation (Figure 2A-B). As with the results in the preliminary screenings, the activity of norspermidine required much higher concentrations to achieve a significant effect compared to other previously characterized polyamines, putrescine and spermidine (Goytia et al. 2013; Nesse et al. 2015; Ramon-Perez et al. 2014). As a qualitative confirmation of the above findings, visualization of bacterial biofilms by confocal laser microscopy (CLSM) showed similar effects of

norspermidine on biofilm inhibition and dispersion, with *A. baumannii* and *K. pneumoniae* having the most visually significant changes in the biofilm phenotype (Figure 2C).

Norspermidine inhibits motility and quorum sensing in *A. baumannii*

Among the isolates tested, *A. baumannii* biofilms were more sensitive to the activity of norspermidine. Given the propensity for multidrug resistance among *A. baumannii* isolates, as well as reports indicating biofilm formation by this pathogen may be a risk factor for the development of persistent infections associated with traumatic wounds (Akers et al. 2014), we further evaluated the effects of norspermidine on aspects crucial for *A. baumannii* biofilm formation including bacterial motility and quorum sensing.

In the presence of norspermidine at 5 and 20 mM, migration (i.e. bacterial motility) of all three clinical isolates of *A. baumannii* on 0.3% TSA plates was significantly reduced (Figure 3A-B). For *A. baumannii* isolates 2 and 4, supplementation of TSA plates with 5 and 20 mM norspermidine significantly reduced bacterial motility compared to control groups; whereas for isolate 3, a significant effect on bacterial migration was only observed following exposure to 20 mM norspermidine. In addition to bacterial motility, expression of a gene operon identified in *A. baumannii* strain 17978 known to be related to quorum sensing production in *A. baumannii* (A1S_0109, A1S_0112, A1S_0113, A1S_0114) was slightly decreased (1 fold) compared to untreated controls (Figure 3C). This effect of norspermidine on the expression of genes involved in quorum sensing was also observed in two of the *A. baumannii* clinical isolates (Figure 3D).

Assessment of biocompatibility of norspermidine on whole cell tissues and human cell lines *in vitro*

As an initial assessment for the potential clinical application of norspermidine as a topical agent for wounds, we evaluated the effect of norspermidine on porcine whole tissues. Direct application of norspermidine to whole tissues was observed to have a time- and concentration-dependent detrimental effect on tissue viability. In particular, exposure of tissues to concentrations of norspermidine exceeding 5 mM had the greatest effect, significantly altering tissue histology and reducing tissue viability after 6 and 24h of exposure (Figure 4A-C). In contrast, concentrations at 5mM did not impair tissue viability even following exposures up to 24h. Having observed the effect of norspermidine on whole tissues, we further evaluated its effect on individual cell lines. Consistent with the experiments performed in whole tissues, both a concentration- and time-dependent effect of norspermidine was observed on the individual cell lines (Figure 4D-E). As with the whole tissues, exposure of cell lines, human keratinocytes and fibroblasts to concentrations exceeding 5 mM were associated with significant losses in cell viability, evident at 6 hours and increasing up to 48h. Consistent with the whole tissue experiments, exposure to concentrations below 5 mM were associated with minor reductions in cell viability.

DISCUSSION

Polyamines are small molecular weight, cationic molecules that have been broadly implicated in a number of bacterial processes including biofilm formation. In a recent study,

Kolodkin-Gal et al. speculated that the polyamine norspermidine inhibited biofilm formation by *B. subtilis* (NCB13610), *S. aureus* (SC01), and *E. coli* (MC4100), and dispersed mature biofilms of *B. subtilis* via destabilizing interactions with exopolysaccharide polymers within the biofilm matrix (Kolodkin-Gal et al. 2012). Recent findings have challenged this proposed mechanism, however, instead suggesting a polysaccharide-independent mechanism of action (Hobley et al. 2014). While these studies provide insight into the potential antibiofilm activities of norspermidine, they are limited to evaluating a select few bacterial species; moreover, there has been discourse surrounding variability in concentrations required to exert this effect on bacterial biofilms. To address this, herein we evaluated the inhibitory/dispersive activity of norspermidine using a panel of genetically distinct multidrug-resistant clinical strains of Gram-negative and Gram-positive organisms associated with chronic wound infections. Additionally, we evaluated the biocompatibility of norspermidine using whole tissues and cell lines to determine the potential application of norspermidine as a strategy to control bacterial biofilms. Collectively, we demonstrated that 5–20 mM norspermidine has inhibitory and disruptive activity against biofilms of clinical isolates of various bacterial species, including *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*, with the greatest effect on clinical strains of *A. baumannii*. Importantly, exposure of whole tissues and cell lines revealed a concentration- and time-dependent cytotoxic effect, potentially delineating limits to concentration and/or duration of application for clinical use. To our knowledge, this is one of the few studies evaluating the activity of norspermidine against clinical isolates of Gram-negative and Gram-positive bacteria associated with chronic human infections and is the first to evaluate the biocompatibility as an initial assessment of its potential clinical utility.

Norspermidine was observed to have anti-biofilm, and in some instances antimicrobial, activity against the bacterial species we evaluated herein. The activity of norspermidine was observed to be both species- and strain-dependent, with *A. baumannii* isolates being the most sensitive to these effects. Strain- and species-dependent differences in the response of isolates to norspermidine are consistent with previous studies and have been previously documented (Nesse et al. 2015; Ramon-Perez et al. 2014). This may be due differences between strains in their ability to develop biofilms on abiotic surfaces, differences in the composition of polymeric matrix, and undefined mechanisms impeding biofilm formation or dispersal initiation. Notably, while anti-biofilm activity was observed with norspermidine, this activity required much higher concentrations compared to putrescine and spermidine (Goytia et al. 2013; Nesse et al. 2015; Ramon-Perez et al. 2014). Because other polyamines have similar chemical properties and are suggested to have similar mechanisms of biofilm disruption to norspermidine, given the specific activity on biofilm but not planktonic growth, it is possible that relative differences may arise from differences in solubility, and/or other undefined mechanisms of activity, for the species evaluated herein.

With the exception of *S. aureus*, the antimicrobial activity of norspermidine was largely independent of planktonic bacterial growth inhibition, and was specific to biofilms. Our observations of a limited effect of norspermidine on bacterial cell growth for most species contrast with previous findings reported by Hobley et al., who noted significantly impaired growth of *B. subtilis* at concentrations ≥ 1 mM (Hobley et al. 2014). Our findings showing minimal effect of norspermidine on planktonic growth of the Gram negative species

evaluated herein are consistent previous studies evaluating the effect of norspermidine at concentrations up to 5 mM on the planktonic growth of *E. coli* and *Salmonella enterica* (Nesse et al. 2015). Similarly, studies evaluating the activity of the related polyamine, spermine, on biofilms of *Neisseria gonorrhoeae* have reported that concentrations as high as 4 mM inhibited biofilm formation but had no notable effect on bacterial growth (Goytia et al. 2013). In contrast, exposure of *S. aureus* to norspermidine at 20 mM was associated with a negative effect on cell growth. While the antimicrobial mechanism is not currently known, it is possible that susceptibility to norspermidine of Gram-positive organisms, such as *B. subtilis* and *S. aureus*, would differ from Gram-negative organisms.

The concentrations of norspermidine required for activity observed in our study are much higher than those reported by Kolodkin-Gal et al., who reported activity with concentrations as low as 25 μ M for *B. subtilis*, and between 100 – 500 μ M for *S. aureus* and *E. coli* for biofilm inhibition (Kolodkin-Gal et al. 2012). Although we did not evaluate their strains, for *S. aureus* we observed activity only at norspermidine concentrations 5 mM (i.e., a ~10 - 40 fold difference). Recent studies evaluating the antibiofilm activity of norspermidine and other polyamines against clinical isolates have demonstrated that higher concentrations are required for biofilm inhibition. In one such study, concentrations of norspermidine between 500-5000 μ M were required to inhibit biofilms of clinical isolates of *E. coli*, (Nesse et al. 2015) whereas the inhibitory activity of spermine on *N. gonorrhoeae* biofilm formation required concentrations as high as 4 mM (Goytia et al. 2013). Aside from inhibition of biofilm formation, dispersal of biofilms was also observed. Although Kolodkin-Gal et al. showed that norspermidine was capable of dispersing biofilms of *B. subtilis*, their study did not evaluate its dispersive activity against *S. aureus* or *E. coli* (Kolodkin-Gal et al. 2012).

Importantly, we are not the first to observe discrepancies in potency compared to the report of Kolodkin-Gal et al. (Hobley et al. 2014). These differences between studies have been attributed to differences in strain and/or species; however they may also relate to the use of strongly alkaline (high pH) commercial preparations of norspermidine. To eliminate the antimicrobial effect of pH, we neutralized norspermidine solutions with HEPES buffer to near neutral pH (7-7.4) before experimental use. This important step was not mentioned in the methods of the original study by Kolodkin-Gal et al., and subsequent studies using neutral preparations have demonstrated the need for higher concentrations of norspermidine (Goytia et al. 2013; Hobley et al. 2014; Nesse et al. 2015; Ramon-Perez et al. 2014). Despite methodological differences making comparisons difficult, numerous reports indicate that norspermidine and other polyamines do have antibiofilm activity, and may ultimately prove to be clinically useful to regulate biofilm formation.

A. baumannii isolates were most susceptible to the effect of norspermidine among the various species tested. Recently, *A. baumannii* has received significant attention for its propensity to acquire resistance to multiple antibiotics and its ability to cause a spectrum of severe infections (Corbella et al. 2000; Dijkshoorn et al. 2007; Gordon and Wareham 2010; Munoz-Price and Weinstein 2008; Peleg et al. 2008). Additionally, the ability of *A. baumannii* to develop biofilms has been implicated as an important pathogenic mechanism (Gurung et al. 2013; Lee et al. 2008; Rodriguez-Bano et al. 2008; Sanchez et al. 2013; Wand et al. 2012), and identified as a potential risk factor for the development of persistent wound

infections (Akers et al. 2014). A major component of *A. baumannii* biofilm matrices, similar to that described for the staphylococcal species, are polysaccharides, in particular poly- β -1,6-N-acetylglucosamine (Bentancor et al. 2012; Choi et al. 2009). Because the main mode of action described for norspermidine was the interference of interactions between polysaccharides central to the development of a stable biofilm matrix (Kolodkin-Gal et al. 2012), interface with the polysaccharide components could in part explain the potent activity observed herein. This is consistent with previous reports involving *S. epidermidis* biofilms (Ramon-Perez et al. 2014). However, exposure of *A. baumannii* isolates to norspermidine also reduced bacterial motility and the expression of genes involved in the synthesis of the quorum sensing signal, acyl-homoserine lactone (ACH), potentially contributing to the observed potency of norspermidine in these isolates (Rumbo-Feal et al. 2013; Saroj and Rather 2013).

Pili-like structures have been shown to contribute to the initial steps of bacterial attachment and formation of biofilms on abiotic surfaces (Gaddy and Actis 2009; Longo et al. 2014). The pili-like structures, including fimbriae encoded by the *csuA/BABCDE* gene and the type IV pilus, are involved in different stages of *Acinetobacter* biofilm development, and are widely distributed among clinical strains (Nait Chabane et al. 2014; Tomaras et al. 2003). The general loss of bacterial motility observed herein, which is largely dependent on these structures, are similar to findings in a previous study evaluating the effect of virstatin, a small organic molecule capable of targeting virulence factors including the type I and type IV pili (Cegelski et al. 2009; Clemmer et al. 2011; Hung et al. 2005). In this study, virstatin was shown to inhibit bacterial motility and consequently decrease in biofilm formation by clinical isolates of *A. baumannii*, in part through the activities on pili biogenesis (Nait Chabane et al. 2014). Although we did not directly evaluate the effect of norspermidine on pili biogenesis, our findings do suggest a potential role of norspermidine on motility.

Interestingly, the expression of genes involved in the synthesis of quorum signaling molecules was also diminished following exposure to norspermidine. Biofilm formation for many bacteria, including *A. baumannii*, is mediated through quorum-sensing pathways (Gaddy and Actis 2009; Longo et al. 2014; Rodriguez-Bano et al. 2008). Surface motility for *A. baumannii* is regulated by diverse mechanisms, including quorum sensing (Clemmer et al. 2011; Saroj and Rather 2013). Previous studies evaluating this relationship have shown that strains of *A. baumannii* deficient in the genes responsible for quorum signal production were also hindered in motility as well as biofilm formation (Clemmer et al. 2011). Collectively, our findings indicate that norspermidine has multiple effects on biofilms independent of interactions with polysaccharides. For *A. baumannii*, the loss in motility and subsequently biofilm formation could in part have been due to interference with quorum sensing. Future studies evaluating the effects of similar aspects of biofilm formation for other bacterial species are necessary.

As a preliminary evaluation of the potential clinical application of norspermidine, we evaluated the associated toxicity on whole tissues as well as individual cell lines following exposure to norspermidine for specified periods of time. Both a concentration- and time-dependent cytotoxic effect of norspermidine was observed within tissues as well as on individual cells lines following exposure to concentrations of norspermidine of 20 mM at

times exceeding 6 hours. Exposure of tissues beyond 6 hours was associated with significant changes in tissue histology, as well as a significant increase in the number of apoptotic cells. Likewise, when evaluated using individual cell lines of keratinocytes and fibroblasts, we observed similar negative effects on viability. Given that the optimal biofilm dispersive and inhibitory activities of norspermidine were observed at higher concentrations, our findings indicate that direct application of norspermidine may incur a risk of local tissue toxicity, particularly when the higher concentrations are used for extended periods. However, it is important to note that while these *in vitro* findings suggest high tissue toxicity, similar findings have been reported for other commonly used wound irrigants, such as Dakin's Solution (buffered sodium hypochlorite) for which clinical use is well accepted (Siala et al. 2014). While host tissue toxicity may limit prolonged, direct application of norspermidine, further studies evaluating brief exposure to higher concentrations, and/or prolonged exposures (>24 hr) to lower biocompatible concentrations, are warranted. Additionally, further studies examining the potentiating effect of topical norspermidine on systemic antimicrobials (Siala et al. 2014) may be helpful to inform the potential clinical utility of norspermidine as an adjunctive treatment for wound infections.

CONCLUSIONS

Biofilm formation is recognized as a significant pathogenic mechanism contributing to the development of chronic human infections. Herein, we demonstrate that the polyamine norspermidine has inhibitory and dispersive activity against clinical isolates of Gram-positive and Gram-negative multidrug-resistant bacteria obtained from persistent human infections. Norspermidine appears to be particularly potent against biofilms of *A. baumannii*, presumably due to multiple mechanisms of biofilm inhibition. Our *in vitro* assessment suggests a potential risk for tissue toxicity from norspermidine at higher concentrations (5-20 mM), with more favorable biocompatibility at concentrations below 5 mM. While this may limit its suitability as a topical anti-biofilm agent, further studies should explore treatment strategies using norspermidine concentrations below 5 mM, with or without conventional antibiotics, to optimize the treatment of biofilm-related bacterial wound infections.

METHODS

Bacterial strains and culture conditions

Bacterial isolates used in this study were prospectively collected from wound infections, as defined by criteria of the U.S. National Healthcare Safety Network, of the upper and lower extremities of injured U.S. military personnel as part of the Trauma Infectious Disease Outcomes Study (**Table 1**) (Akers et al. 2014; Tribble et al. 2011). All isolates were originally collected from patients as a part of treatment and not related to research. All isolates were previously determined to be phenotypically positive for biofilm formation according to the crystal violet assay (Akers et al. 2014; Sanchez et al. 2013). Isolates were considered to be associated with persistent infections if they were recovered from the same anatomic site 14 days after the initial isolate of the same species. For this study, only isolates associated with persistent wound infections were selected to ensure that strains

evaluated were likely to be biofilm producers *in vivo*, as well as *in vitro*, given the chronicity of infection. Organisms were defined as multidrug-resistant if they exhibited resistance to at least three of the major antibiotic classes (aminoglycosides, β -lactams, carbapenems, and fluoroquinolones) or produced either extended spectrum β -lactamases or *K. pneumoniae* carbapenemases. Bacterial isolates were recovered from frozen storage at -80°C and sub-cultured on blood agar plates (Remel, Lenexa, KS) overnight at 37°C prior to each experimental assay. Bacterial cultures were grown in cation adjusted Mueller-Hinton Broth (MHB-II) with agitation at 37°C .

Reagents and preparation of norspermidine

Norspermidine (bis-(3-aminopropyl)-amine), putrescine, and spermidine were purchased from Sigma-Aldrich (St. Louis, MO). For use in experimental assays, norspermidine was diluted into MHB-II containing 100 mM HEPES buffer, to maintain a near-neutral pH, at the tested concentrations. Putrescine and spermidine were diluted in sterile water per the recommendations of the manufacturer.

Biofilm formation and dispersal/inhibition assays

Biofilm formation was examined under static conditions using the semi-quantitative 96-well plate (Corning, Inc., Corning, NY, USA) biofilm model as previously described (Christensen et al. 1985). Briefly, overnight bacterial cultures were diluted 1:100 in fresh MHB-II and individual wells were filled with 180 μL and incubated at 37°C for 24 h. Following overnight incubation, plates were gently washed with 1X phosphate buffered saline (PBS; pH 7.4) to remove non-adherent bacteria and stained with 100 μL of 0.1% (w/v) crystal violet (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. Excess crystal violet was removed by washing with PBS, and resulting biofilm was quantified by measuring the corresponding OD₅₇₀ nm of the dye following solubilization in 70% ethanol (v/v). Biofilm biomass was determined by measuring the absorbance of solubilized stain at 570 nm using a microtiter plate reader.

To assess the biofilm dispersal activity of norspermidine, culture medium from biofilms was removed after 24 h, and 200 μL of culture medium supplemented with norspermidine in 100 mM HEPES buffer at the indicated concentrations was added into individual wells with the pre-established biofilm. After overnight treatment, plates were gently washed with PBS to remove unattached cells, stained with crystal violet, rinsed, and then solubilized with ethanol as above. For assays evaluating the ability of norspermidine to inhibit biofilm formation, cells were grown as described above in the presence of media containing norspermidine at the indicated concentrations. Dispersive and inhibitory activity of norspermidine was reported as a percentage dispersal (or inhibition) relative to the untreated control group, exposed to media containing 100 mM HEPES buffer. For each clinical strain tested, biofilm assays were performed in triplicate with a minimum of three replicates per experiment, and the mean biofilm absorbance value of the replicates was determined.

Confocal Scanning Laser Microscopy (CLSM)

Bacterial biofilms were visualized using an Olympus FluoView confocal laser scanning microscope (Olympus, Pittsburgh, PA). Briefly, biofilms were grown as described above

using glass chamber slides, as previously described (Cardile et al. 2014). Following overnight treatment (inhibition or dispersal), biofilms were gently washed with sterile PBS, fixed for 10 min at room temperature with 4% paraformaldehyde in PBS, and stained using Film Tracer™ SYPRO® Ruby Biofilm Matrix Stain, according to the manufacturer's instructions (Molecular Probes, Eugene, OR). CLSM images were acquired at 20X magnification using an argon laser (488 nm). Image analysis and Z-stacks were acquired using the Olympus FluoView software. Images were taken from three distinct regions on the slide, and representative images were selected for each treatment group.

Motility assays

Bacterial motility was analyzed as previously described on 0.5% agar TSB plates supplemented with 5 g/L tryptone, 2.5 g/L NaCl and 100 mM HEPES, pH 7 (Nait Chabane et al. 2014; Skiebe et al. 2012). Briefly, single bacterial colonies cultured on blood agar plates were selected with a sterile pipet tip. This tip was then used to puncture the agar to inoculate bacteria directly into the motility plate. Motility was analyzed after 24 h of growth at 37°C, by measuring the radius, in millimeters (mm), of the motility zone of bacteria growing between the bottom of the agar layer and the polystyrene petri dish. Assays were repeated in triplicate.

RNA isolation and quantitative real time reverse transcription PCR (qRT-PCR)

To evaluate the effect of norspermidine on the expression of genes related to homoserine lactone synthesis (A1S_0109 - A1S_0114) in *A. baumannii*, including a reference strain ATCC 17978 as well as three representative clinical isolates, biofilms were formed in 6-well tissue culture plates using previously described methods in the presence of 5 and 20 mM norspermidine (in 100mM HEPES) for 24 h at 37°C (Cardile et al. 2014; Malachowa et al. 2011). Following overnight incubation, supernatants were removed, individual wells were gently washed with PBS and attached bacteria (i.e. biofilm bacteria) were detached from wells by scraping with pipette tips and resuspended in 500 µL of PBS. Bacteria were harvested by centrifugation, washed, lysed, and the RNA was isolated using PureLink RNA Mini Kit (Life Technologies, Grand Island, NY) following treatment of samples with Bacterial-RNA Protect (Qiagen, Valencia, CA) as recommended by the manufacturer. Isolated RNA was reverse transcribed using the High Capacity cDNA reverse transcription kit (Life Technologies), per the manufacturer's protocol. Quantitation of gene expression was via TaqMan (Life Technologies) methodology using the relative standard curve method. The gene-specific PCR primers (Table 2) were developed with Primer Express software (Life Technologies). Real-time quantitative PCR reactions consisted of the cDNA template (1.5 ng), 1X Universal PCR Master Mix for Gene Expression (Life Technologies), gene specific primers (900 nM) and probe (250 nM) in a total volume of 20 µL. Standard curves consisted of ten-fold dilutions of a positive control sample. PCR reactions were performed in triplicate and cycled in a 7900HT Sequence Detection System using standard protocols (Applied Biosystems, Grand Island, NY). Transcript levels were normalized to the expression levels of the internal control mRNA, Gyrase B (*gyrB*) and fold-regulation changes was calculated using 2^{-C_t} method.

Tissue Viability Evaluation

Tissues used in this study were harvested from the dermis of Sinclair Miniature pigs (*Sus scrofa*) which was provided post-mortem as part of a tissue sharing agreement and in compliance with an approved animal study protocol through the Animal Welfare Act and the implementing Animal Welfare Regulations. For the experiments in this study, no direct animal experimentation was involved. Briefly, porcine skin explants were extracted from tissues using a 6 mm biopsy punch. Tissues were washed with PBS and then sterilized by 15 min exposures to 70% ethanol solution followed by 0.615% sodium hypochlorite solution prepared in PBS (Yang et al. 2013). Biopsied tissues (n=6) were then exposed to norspermidine at the designated concentrations diluted in saline with 100 mM HEPES buffer for 24 h. Following exposure, tissues were washed twice with PBS, transferred to a 96-well plate, and incubated with MTT cell viability reagent (ATCC, Manassas, VA) for 2 hr at 37°C. Tissues were then transferred to individual wells of a 96-well plate containing dimethyl sulfoxide (0.07 mL) and incubated for an additional 1 hr at 37°C to extract the MTT reagent. Solubilized formazan was quantified by measuring the absorbance at 540 nm. As a positive control for the MTT assay, a group of tissue samples were heated to 200°C for 5 min. The average of optical density values of the positive control were then subtracted from the absorbance values obtained for all other samples. Tissue viability of explants was expressed as the ratio between the observed OD_{540nm} and the weight of the explant in grams, and reported as a percentage to non-treated control as previously described (Castagnoli et al. 2003).

Histological analysis and immunohistochemistry

Excised tissue sections were fixed in 10% neutral buffered formalin for 48 hr, embedded in paraffin, and then cut into 7 µm cross-sectional slices. Slides were deparaffinized in xylene, rehydrated in water and stained with Weigert's iron hematoxylin solution kit (Sigma Aldrich, St. Louis, MO). Immunohistochemistry was performed as follows: heat-mediated antigen retrieval with 0.01M citrate buffer at 95-98°C for 15 min, blocking with 10% horse serum in Hanks' balanced salt solution (HBSS) for 30 min at room temperature, followed by incubation with a 1:300 dilution of rabbit polyclonal antibody to caspase-3 (Abcam, Cambridge, MA) overnight at 4°C. Following overnight incubation, slides were washed with HBSS and exposed to horse anti-rabbit biotinylated secondary antibody for 60 min at room temperature (Vector Labs, Burlingame, CA). Tissue sections were then incubated with Vectastain-RTU Kit solution and ImmPACT DAB Diaminobenzidine (Vector Laboratories) followed by counterstaining with hematoxylin and dehydration prior to cover slipping. Representative images of H&E and caspase-3 stained tissue sections were captured at 10 and 20 X magnifications using a Zeiss AxioScan Z1 slide scanner (Munich, Germany).

Cell Lines and Viability Assays

Human epidermal keratinocytes (HEK001; ATCC CRL-2404; ATCC, Manassas, VA, USA) were grown in keratinocyte serum-free medium (GIBCO, Grand Island, NY) supplemented with 5 ng/ml of human recombinant epidermal growth factor (EGF) and 2 mM of L-glutamine. Human dermal fibroblasts (PromoCell, Heidelberg, Germany) were grown in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, 10 U/ml of

penicillin, and 10 µg/ml of streptomycin. Cell lines were grown and maintained at 37°C in 5% carbon dioxide. Cellular viability assays were performed as previously described (Barsoumian et al. 2013), which in brief consisted of exposing confluent monolayers of cells to norspermidine (1, 5, 25 mM in 100mM HEPES buffer (pH 7)) diluted into the appropriate cell medias for up to 24 h at 37°C in 5% CO₂. Following exposure, cells were washed, resuspended in phosphate buffered saline (pH 7.4), and cell viability measured using Cell Titer-Fluor assay (Promega, Madison, WI, USA) as recommended by the manufacturer. As a negative control, cells were exposed to media containing only 100 mM HEPES buffer and viability reported as a percentage of the non-treated control group. Assays were performed at least twice with a minimum of three technical replicates per test condition.

Statistical analysis

Where appropriate, statistical analysis was performed using an unpaired, two-tail student's *t* test or a One-way ANOVA with a Dunnett's post-hoc evaluation for comparison of the control group between multiple treatment groups. *P* values of <0.05 were considered to be statistically significant.

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Figure 1. Screening of the antibiofilm and antimicrobial activity of norspermidine on bacterial isolates

Screening of the dispersive (A) and inhibitory (B) activity of norspermidine, at concentrations ranging from 0.1 mM to 20 mM in HEPES buffer (pH ~7.4), putrescine (0.1 mM) and spermidine (0.1 mM) against preformed biofilms (A) or following overnight incubation in the presence of norspermidine (B) against four representative clinical isolates of *A. baumannii* (AB1), *K. pneumoniae* (KP1), *P. aeruginosa* (PA1), and *S. aureus* (SA1) (described in Table 1). Biomass was determined using the crystal violet method and reported as the percentage of biofilm dispersal and inhibition to untreated controls. C) Planktonic bacterial growth up to 24 h in liquid media supplemented with norspermidine (0.1-20 mM), putrescine (0.1 mM) and spermidine (0.1 mM), as determined by measuring the optical density at 600 nm and plotted over time. Bars/lines represent the average $\pm$ SD of three independent experiments. Statistical analysis was performed using a student's *t* test; $p < 0.05$ was considered to be statistically different from the untreated control group.

Figure 2. Norspermidine disperses biofilms and prevents biofilm formation in clinical isolates associated with persistent infections

Dispersive (A) and inhibitory (B) activity of norspermidine at 5 and 20 mM in HEPES buffer (pH ~7.4) against preformed biofilms or following overnight incubation in the presence of norspermidine (NrSp), 5 and 20 mM, putrescine (0.1 mM) or spermidine (0.1 mM) against clinical isolates of bacteria associated with persistent infections (described in Table 1). Biomass was determined using the crystal violet method, and reported as the percentage of biofilm dispersal and inhibition to untreated controls. (C) Representative CLSM images of the biofilm dispersive and inhibitory activity of norspermidine at 20 mM on the various bacterial species. Bars represent the average $\pm$ SD of three independent experiments. Statistical analysis was performed using a student's t test; $p < 0.05$ was considered to be statistically different from the untreated control group.

Figure 3. Effect of norspermidine on bacterial motility and quorum sensing in *Acinetobacter baumannii*

A) Representative images of semi-solid 0.3% TSA plates with or without norspermidine (NrSP) (5 and 20 mM) after 24 h growth at 37°C demonstrating the motility of *A. baumannii* strains. B) bacterial motility, as determined by measuring the diameter of the motility zone (the difference between white and black dashed lines in Fig. 3A) in mm. C-D) comparison of the expression levels of genes related to homoserine lactone synthesis (see Table 2) in *A. baumannii* reference strain 17978 (Fig. 3C) and clinical strains (Fig. 3D) as determined by qRT-PCR assays following exposure to norspermidine. Bars represent the average $\pm$ SD of three independent experiments. Statistical analysis was performed using an ANOVA One-Way; $p < 0.05$ was considered to be statistically different from the untreated control group.

Figure 4. Assessment of the biocompatibility of norspermidine *In vitro*

In vitro evaluation of the effect of norspermidine, 1, 5, and 20 mM, on whole tissue porcine explants following overnight exposure. A-B) Representative images (10X; 20X inset) of norspermidine-treated porcine tissues as evaluated by H&E staining (Fig. 4A) and immunohistochemistry using an antibody to activated caspase-3 (Fig. 4B). C) Cell viability of tissues as determined using the MTT viability assays and reported as an index of viability relative to the control (media only) group. A heat-treated group was included as a negative control. D-E) Bars represent the average $\pm$ SD of three independent experiments. Values above the bars represent the % viability loss for each group. Statistical analysis was performed using an ANOVA One-Way; $p < 0.05$ was considered to be statistically different from the untreated control group.

TABLE 1

Characteristics of Bacterial Strains Used in this Study

Bacterial Species	Isolate ^a	Source	Specific Site	Phenotype ^b	Pulse Field Type (PFT)	Days From Initial Isolate	Biofilm Former
<i>Acinetobacter baumannii</i>	AB 1	Wound	Lower Leg	MDR		0	Y
	AB 2	Wound	Forearm	MDR	7	20	Y
	AB 3	Wound	Lower Leg	MDR	50	21	Y
	AB 4	Wound	Upper Arm	MDR	44	24	Y
<i>Klebsiella pneumoniae</i>	KP 1	Wound	Thigh	MDR		0	Y
	KP 2	Wound	Thigh	MDR	68	44	Y
	KP 3	Wound	Upper Arm	MDR	67	15	Y
<i>Pseudomonas aeruginosa</i>	PA 1			MDR			Y
	PA 2	Wound	Forearm	MDR	26	14	Y
	PA 3	Wound	Thigh	MDR	25	47	Y
	PA 4	Wound	Thigh	MDR	29	16	Y
	PA 5	Wound	Thigh	MDR	32	17	Y
<i>Staphylococcus aureus</i>	SA 1	Wound	Bone	MSSA	USA 200	-	Y
	SA 2	Wound	Foot	MRSA	USA 100	206	Y
	SA 3	Wound	Foot	MRSA	17	20	Y
	SA 4	Wound	Knee	MRSA	16	91	Y

^a Isolates collected from wound infections of the upper and lower extremities of injured U.S. military personnel as part of the Trauma Infectious Disease Outcomes Study (Tribble et al. 2011, Akers et al. 2014).

^b A multidrug-resistant (MDR) organism was defined as an organism resistant to antimicrobials in 3 or more classes of antimicrobial agents (penicillins/cephalosporins, carbapenems, aminoglycosides, and quinolones) not including tetracyclines or colistin. MSSA/MRSA: methicillin-susceptible/-resistant *S. aureus*.

TABLE 2

Quantitative real-time PCR (qRT-PCR) Primers and Probes Used In this Study

Gene	Primer/Probe Name	Sequence
DNA gyrase	A1S_0004-F	5'-GATGATGCGCGTGAAGGTTT-3'
	A1S_004-R	5'-GACGAGAATTCGGATCAGGAA-3'
	A1S_004 PROBE	5'-ACAGCCATTATTCTG-3'
homoserine lactone synthase	A1S_0109-F	5'-GATTTTCAAATCCGCCTTCCT-3'
	A1S_0109-R	5'-TGCAATTGAGACCGGTGATG-3'
	A1S_0109 PROBE	5'-AGCAGTCAGGCTGTG-3'
acyl-CoA synthetase	A1S_0112-F	5'-GATCGGCTCGGTTTTGCA-3'
	A1S_0112-R	5'-GCGGCGACATGACATAACAA-3'
	A1S0112 PROBE	5'-ACCGTATATGTGGGACTGA-3'
acyl-CoA dehydrogenase	A1S_0113-F	5'-TCGCTACAGCTTATGCCTACCTT-3'
	A1S_0113-R	5'-TCGCGCACCAACAAGTGA-3'
	A1S_0113 PROBE	5'-TAGCGGCTGAGGTGTT-3'
acyl-carrier protein	A1S_0114-F	5'-AAGCTTACTGGAGCGCAATCA-3'
	A1S_0114-R	5'-CAGGCTCTACGCGCATCTCT-3'
	A1S_0114 PROBE	5'-TCGTACGCTAGTCGCT-3'