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Short Communication

Nitroxoline: a broad-spectrum biofilm-eradicating agent against pathogenic bacteria

Yasmeen Abouelhassan [a], Qingping Yang [b], Hussain Yousaf [a], Minh Thu Nguyen [a], Melanie Rolfe [a], Gregory S. Schultz [b], Robert W. Huigens III [a,*]

[a] Department of Medicinal Chemistry, College of Pharmacy, University of Florida, 1600 SW Archer Road, Gainesville, FL 32610, USA
[b] Institute of Wound Research, Department of Obstetrics and Gynecology, College of Medicine, University of Florida, Gainesville, FL 32610, USA

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ABSTRACT

Bacterial biofilms are surface-attached communities of slow-growing or non-replicating bacteria tolerant to conventional antibiotic therapies. Although biofilms are known to occur in ca. 80% of all bacterial infections, no therapeutic agent has been developed to eradicate bacteria housed within biofilms. We have discovered that nitroxoline, an antibacterial agent used to treat urinary tract infections, displays broad-spectrum biofilm-eradicating activities against major human pathogens, including drug-resistant *Staphylococcus aureus* and *Acinetobacter baumannii* strains. In this study, the effectiveness of nitroxoline to eradicate biofilms was determined using an in vitro [minimum biofilm eradication concentration (MBEC) = 46.9 μM against *A. baumannii*] and ex vivo porcine skin model (2–3 log reduction in viable biofilm cells). Nitroxoline was also found to eradicate methicillin-resistant *S. aureus* (MRSA) persister cells in non-biofilm (stationary) cultures, whereas vancomycin and daptomycin were found to be inactive. These findings could lead to effective, nitroxoline-based therapies for biofilm-associated infections.

1. Introduction

Free-floating planktonic bacteria use a signalling process known as quorum sensing to co-ordinate virulent behaviours, including the formation of surface-attached biofilms [1]. Bacteria encased within a biofilm lead a slow- or non-growing (dormant) existence and have contrastingly different gene expression profiles compared with their planktonic counterparts [2–4]. As a result, biofilms are highly tolerant to conventional antibiotics and are the underlying cause of many recurring and chronic infections [5].

We have discovered halogenated quinolines and phenazines capable of eradicating biofilms of multiple Gram-positive pathogens [6–10], which led us to nitroxoline, a structurally related antibacterial agent used to treat urinary tract infections [11,12]. Nitroxoline operates through a metal chelation-dependent mechanism and has reported biofilm dispersal activity against *Pseudomonas aeruginosa* [13]. Here we report our findings of the broad-spectrum biofilm-eradicating activities of nitroxoline against multiple human pathogens. This phenotype is extremely rare as mitomycin C has been the only reported broad-spectrum biofilm-eradicating agent that does not target bacterial membranes for destruction [14].

2. Materials and methods

2.1. Bacterial strains

Multidrug-resistant (MDR) *Acinetobacter baumannii* ATCC 1794, *A. baumannii* ATCC 19606, *P. aeruginosa* PAO1, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC BAA-1707, methicillin-resistant *Staphylococcus epidermidis* (MRSE) ATCC 35984 and vancomycin-resistant *Enterococcus faecium* (VRE) ATCC 700221 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Methicillin-resistant *S. aureus* MRSA-1, MRSA-2 and MRSA-156 and methicillin-resistant *S. epidermidis* MRSE-1 were obtained as clinical isolates from patients treated at Shands Hospital (Gainesville, FL). UAEC-1 is an *Escherichia coli* isolate from UAMS Hospital (Fayetteville, AR).

2.2. Minimum inhibitory concentration (MIC) assays

MIC assays were performed as previously described to determine antibacterial activities against pathogenic bacteria [6–10,15]. Results from MIC assays were obtained from three independent experiments (Table 1).

2.3. Minimum biofilm eradication concentration (MBEC) assays

Biofilm eradication assays were performed in 96-well plates (polystyrene for MRSA-2 and MRSE-1; polyvinyl chloride for *A.*

* Corresponding author. Department of Medicinal Chemistry, College of Pharmacy, University of Florida, 1600 SW Archer Road, Gainesville, FL 32610, USA.
E-mail address: rhuigens@cop.ufl.edu (R.W. Huigens III).

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Table 1
Summary of antibacterial and biofilm eradication assays performed in 96-well plates and in Calgary Biofilm Devices (CBDs). All values are reported in μM.

Strain/isolate	Nitroxoline		Colistin[a]		Vancomycin[a]	
	96-well plates (MIC/MBEC)	CBD (MBC/MBEC)	96-well plates (MIC/MBEC)	CBD (MBC/MBEC)	96-well plates (MIC/MBEC)	CBD (MBC/MBEC)
Acinetobacter baumannii 19606[b]	6.25/46.9[c]	–[d]	0.39/1000	–	–	–
A. baumannii 1794[b]	4.69[c]/62.5	125/46.9[c]	0.39/–	375[c]/375[c]	–	–
UAEC-1	12.5/–	93.8[c]/62.5	0.39/–	11.7[c]/46.9[c]	–	–
PAO1	250/1500[c]	–	0.78/1500[c]	–	–	–
MRSA-1	12.5/–	250/188[c]	–	–	0.39/–	15.6[e]/750[c]
MRSA-2[f]	25/188[c]	250/62.5	–	–	0.78/>2000	11.7[c]/62.5
MRSA-156	12.5/–	750[c]/125[c]	–	–	0.78/–	31.3/750[c]
MRSA-BAA-1707	9.38[c]/–	250/93.8[c]	–	–	0.39/–	23.5[c]/1500[c]
MRSE-1[f]	18.8[c]/125	–	–	–	0.59[c]/>2000	–
MRSE 35984	18.8[c]/–	500/250	–	–	–	–
VRE 700221	125/–	125/62.5	–	–	>100/–	>100/150[c]

MIC, minimum inhibitory concentration; MBEC, minimum biofilm eradication concentration; MBC, minimum bactericidal concentration.
All data were obtained from three independent experiments.
[a] Colistin served as the anti-Gram-negative comparator antibiotic and vancomycin served as the anti-Gram-positive comparator antibiotic in these experiments.
[b] *A. baumannii* biofilms were grown on polyvinyl chloride plates.
[c] Corresponds to the mid-point of a two-fold range in MIC, MBC or MBEC values.
[d] Indicates not tested.
[e] Corresponds to the mid-point value of a four-fold range in MBC experiments.
[f] MRSA-2 and MRSE-1 biofilms were grown on polystyrene plates.

baumannii and PAO1) [6] or in Calgary Biofilm Devices (CBDs) [7–10,16]. Biofilm eradication assays involve three phases separated by wash steps, including (i) initial biofilm establishment on well/peg surfaces without test compound; (ii) biofilm treatment with test compound; and (iii) recovery of viable biofilms in fresh medium alone. Both assays were used to demonstrate the biofilm eradication activities of nitroxoline.

For polystyrene and polyvinyl chloride 96-well plate biofilm eradication assays, microtitre wells were inoculated with 100 μL of a 1:1000-fold exponential-phase culture [optical density at 600 nm (OD_{600}) of ca. 1.0] and were incubated for 24 h at 37 °C to allow biofilms to establish on the surface of microtitre wells (phase 1). Following biofilm establishment, medium and planktonic cells were removed from microtitre plates and the plates were rinsed with water. Then, 100 μL of two-fold serial dilutions of test compound was added to the microtitre wells in fresh medium and was incubated for 24 h at 37 °C (phase 2). After this time, the contents of the microtitre wells were removed and 100 μL of fresh medium only was added to allow viable biofilms to recover and to disperse planktonic bacteria into the medium resulting in a turbid microtitre well (24-h incubation at 37 °C; phase 3). After this final phase, microtitre plates were examined for visible bacterial growth (turbidity) and the MBEC was recorded as the lowest concentration at which no turbidity could be observed (due to eradicated biofilms). For MRSA-2 and MRSE-1 biofilm eradication assays, 100 μL of a 1% gelatin (aqueous) solution was used to pre-treat polystyrene plates for a minimum of 1 h to enhance *S. aureus* and *S. epidermidis* biofilm formation, as previously reported [17,18].

Biofilm eradication assays involving CBDs (Innovotech Inc., Edmonton, AB, Canada) (Supplementary Fig. S1) were performed in an analogous manner to the previous microtitre plate assay; however, CBDs allow biofilms to be established and treated on pegs suspended from 96-well plate lids. We have previously reported these assay conditions [7–10,16]. CBD experiments with *A. baumannii* 1794, UAEC-1, all MRSA strains and MRSE 35984 were carried out on hydroxyapatite-coated P&G devices, whilst experiments with VRE 700221 were performed on non-coated P&G CBDs.

2.4. Ex vivo porcine skin biofilm model

Biofilms were formed on porcine skin explants as previously described [17,18] (Supplementary Fig. S2) with slight modifications. Briefly, porcine skin explants were inoculated with 20 μL of bacterial culture (OD_{600} = 0.2–0.4) and were incubated on agar for 48 h at 37 °C to establish biofilms on porcine skin explants. Following biofilm establishment, porcine skin explants were treated with test compound in phosphate-buffered saline (PBS) at 400 μM for 72 h (at 37 °C). After this time, porcine skin explants were rinsed with PBS to remove planktonic cells and the remaining biofilm cells were removed via sonication from the porcine explants. Subsequent colony counts were performed to determine relative biofilm viabilities between treated and untreated (vehicle-only) samples.

2.5. MRSA-2 stationary cell kill kinetics

MRSA-2 stationary killing was performed as previously described [8]. Briefly, an MRSA-2 overnight culture was diluted in fresh medium and was allowed to grow for 4 h to reach stationary phase. Test compound was then added to stationary cultures and aliquots were removed to perform colony counts to determine CFU/mL (viable bacteria) at pre-determined time points.

2.6. Minimum biofilm inhibitory concentration (MBIC) determination

In PVC 96-well plates, two-fold serial dilutions of test compounds were added in LB medium. Then, 1:1000-fold exponential-phase culture (OD_{600} of ca. 0.8) in LB was added to each well and was allowed to incubate at 37 °C for 24 h. After this time, the contents from the 96-well plates were removed and the wells were rinsed with water, followed by the addition of 120 μL of crystal violet to stain the biofilms (0.1% for *A. baumannii* and 1% for PAO1; 10-min incubation at room temperature). The plates were then rinsed and 120 μL of ethanol was added to dissolve the crystal violet-stained biofilms. Minimum concentrations required to inhibit 80% of biofilm formation ($MBIC_{80}$) were determined (OD_{540}) by comparing compound-treated versus untreated wells and the resulting data were used to generate dose–response curves using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA). Note: these experiments were performed to determine whether the antibiofilm activities of nitroxoline were dependent on or independent of their antibacterial activities (Supplementary Table S1; Supplementary Fig. S3).