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Targeting biofilms of multidrug-resistant bacteria with silver oxynitrate

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ABSTRACT

A topical antimicrobial, silver oxynitrate (Ag_7NO_{11}), has recently become available that exploits the antimicrobial activity of ionic silver but has enhanced activity because highly oxidised silver atoms are stabilised with oxygen in a unique chemical formulation. The objective of this study was to use a multifaceted approach to characterise the spectrum of antimicrobial and antibiofilm activity of a wound dressing coated with Ag_7NO_{11} at a concentration of 0.4 mg Ag/cm^2. Physiochemical properties that influence efficacy were also evaluated, and Ag_7NO_{11} was found to release a high level of Ag ions, including Ag^{2+} and Ag^{3+}, without influencing the pH of the medium. Time–kill analysis demonstrated that a panel of multidrug-resistant pathogens isolated from wound specimens remained susceptible to Ag_7NO_{11} over a period of 7 days, even with repeated inoculations of 1×10^6 CFU/mL to the dressing. Furthermore, established 72-h-old biofilms of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and two carbapenem-resistant Gram-negative bacteria (bla_{NDM-1}-positive *Klebsiella pneumoniae* and bla_{VIM-2}-positive *P. aeruginosa*) were disrupted and eradicated by Ag_7NO_{11} in vitro. Ag_7NO_{11} is a proprietary compound that exploits novel Ag chemistry and can be considered a new class of topical antimicrobial agent. Biocompatibility testing has concluded Ag_7NO_{11} to be non-toxic for cytotoxicity, acute systemic toxicity, irritation and sensitisation.

1. Introduction

In 1857, Dr J. Marion Sims declared to the President of the New York Academy of Medicine that 'It is to revolutionize surgical dressings…with silver there is no inflammation, no suppuration' [1]. Since then, silver (Ag) has held a fundamental place in medicine as one of the earliest antimicrobial agents employed to prevent and treat wound infection. Historically, uses of Ag have been limited to its elemental form [$Ag^0_{(s)}$] or as silver nitrate [$AgNO_{3(s)}$], but considering the longstanding use of Ag, innovation in Ag technology has been incremental. One major advance in Ag technology was the introduction of nanocrystalline silver (NCS) dressings in the late 1990s [2,3] that demonstrated significantly greater efficacy than $AgNO_3$ [4]. The increased surface area of NCS allows for enhanced surface oxidation and formation of silver oxide [$Ag_2O_{(s)}$], creating a reservoir of available Ag ions [$Ag^+_{(aq)}$] and release of hydroxide [$OH^-_{(aq)}$] upon contact with an aqueous fluid such as a wound bed. The result is a fast and highly efficacious activity against micro-organisms. The novelty in NCS technology lies in the mechanism of action of Ag itself, the Ag^+ ion is required for antimicrobial activity [5,6] and the physical form of NCS allows for continued release while creating an unfavourable and basic environment for microbes to thrive. The success of NCS dressings led to an explosive increase in the use of Ag dressings for wound care and the development of subsequent technologies.

Like other transition metals, Ag^+ also forms insoluble precipitates [most notably $AgCl_{(s)}$ and $Ag_2SO_{4(s)}$] that are stable and less reactive. It has been demonstrated that Ag chemistry significantly impacts antimicrobial efficacy [7], yet it is these Ag salts that are found in the majority of currently available advanced wound care products, resulting in confounding evidence for the supported use of Ag as a topical antimicrobial [8]. Whilst it is established that the solubility and availability of Ag^+ is the primary driving factor of antimicrobial efficacy, there have been no major developments in the formulation and delivery of Ag to enhance the applicability and antimicrobial activity since the introduction of NCS nearly 20 years ago.

With a dwindling pipeline of new small-molecule antibiotics and the growing threat of resistance, particularly multidrug-resistant (MDR) Gram-negative bacteria such as carbapenem-resistant Enterobacteriaceae (CRE), the pursuit of novel antimicrobials that can target these types of wound infections is one strategy to preserve the use of antibiotics for systemic infection and to minimise the

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Table 1
Log reduction values over 7 days (1×10^6 CFU/mL inoculations occurred every 24 h) of antibiotic-resistant wound isolates exposed to a wound dressing coated with 0.4 mg/cm^2 silver oxynitrate.

Organism	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Stenotrophomonas maltophilia	4.79	7.45	7.78	7.80	7.53	7.84	7.38
Staphylococcus aureus (*mecA*)	4.86	3.56	3.95	3.84	3.55	3.14	4.88
Pseudomonas aeruginosa (bla_{VIM-2})	3.92	7.08	5.98	4.62	5.87	5.21	5.71
Klebsiella pneumoniae (bla_{KPC})	3.89	6.76	5.94	6.17	5.39	5.46	4.70
K. pneumoniae (bla_{NDM-1})	3.31	6.70	6.93	6.44	6.35	6.32	6.41
Escherichia coli	4.60	2.76	3.55	3.98	5.02	5.89	5.24
Enterococcus faecalis (*vanA*)	4.00	5.73	4.93	4.67	4.53	4.82	5.41

spread of resistance. Enhancements to Ag formulations and wound dressings, such as the recent application of the silver oxynitrate [$Ag_7NO_{11(s)}$ or Ag Oxysalt] compound that contains highly oxidised Ag ($Ag^{2+/3+}$) stabilised by oxygen atoms, represents a recent major innovation to Ag technology with enhanced antimicrobial efficacy against MDR pathogens [7,9]. Synthesis of Ag Oxysalt requires deliberate oxidation of Ag^+ and subsequent capture of the crystalline structure as a pure powder that can then be incorporated into a variety of substrates or matrices for topical delivery [9,10]. Upon interaction with fluid, highly unstable Ag^{2+} and Ag^{3+} ions are released, where the difference in the oxidising potential of $Ag^{2+/3+}$ (E_o = 1.98 V and 1.8 V, respectively) compared with that of Ag^+ (E_o = 0.8 V) results in access to different chemistry, as electrons are scavenged from microbial metabolic processes. The minimum biocidal concentration of pure Ag Oxysalt required for killing a broad spectrum of bacteria has been determined to be significantly lower than all other available Ag compounds. Importantly, the minimum concentrations required both to inhibit and eradicate bacteria growing as biofilms is also significantly lower [7].

Here we describe the antimicrobial and antibiofilm activity of a wound contact layer coated with Ag Oxysalt against a spectrum of Gram-positive and Gram-negative bacteria growing planktonically or in a biofilm. We demonstrate that with a 75% reduction of total silver used, the antimicrobial efficacy is equivalent or surpasses a wound dressing containing NCS. Finally, we show that Ag Oxysalt is efficacious against antibiotic-resistant organisms including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and carbapenem-resistant bacteria expressing the bla_{NDM-1}, bla_{VIM-2} and bla_{KPC} genes growing both planktonically and in a biofilm.

2. Methods

2.1. Wound dressings

Commercially available wound dressings were used in this study as follows: Exsalt® T7 and SD7 (Ag Oxysalt dressing, 0.4 mg Ag/cm^2; Exciton Technologies Inc., Edmonton, Canada); Acticoat 7 (NCS 1.2–1.6 mg Ag/cm^2; Smith and Nephew, Hull, UK); Aquacel® Ag and Aquacel® Ag$^+$ Extra™ (AgCl, 0.083–0.09 mg Ag/cm^2; ConvaTec, Greensboro, NC); Mepilex Ag (Ag_2SO_4, 1.2 mg Ag/cm^2; Mölnlycke, Göteburg, Sweden); Silverlon (Ag^0, 5.46 mg Ag/cm^2; Argentum Medical, Geneva, IL); and TheraBond® (Ag^0, 15% Ag w/w; Alliqua, Yardley, PA).

2.2. Bacterial strains

A panel of Gram-negative (*Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and Gram-positive (*Corynebacterium striatum*, vancomycin-sensitive and vancomycin-resistant *Enterococcus faecalis*, *S. aureus* and *Staphylococcus epidermidis*) organisms were supplied by Keystone Labs (Edmonton, Canada). The MRSA isolate tested was isolated from an abscess and was supplied by CanBiocin Inc. (Edmonton, Canada). *Pseudomonas aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 were used for biofilm growth. The antimicrobial-resistant strains listed in Table 1 were originally isolated from cutaneous wounds and were kindly supplied by Dr Robert Rennie (Alberta Provincial Laboratory for Public Health, Edmonton, Canada).

2.3. Time–kill curves and log reduction

Time–kill log reduction analysis was performed at Keystone Labs. Briefly, from overnight cultures, bacteria inocula were made to 1×10^6 CFU/mL in maximum recovery diluent (composed of 0.9% NaCl and 0.1% peptone). Dressings were cut to 2.5 cm × 2.5 cm and were exposed to 5 mL of each suspension. A single reaction tube in duplicate was set up for each time point. After the specified exposure period (0.5, 1, 2, 3 and 4 h), a sample was removed from the reaction tube and was diluted 1/5 into a universal antimicrobial neutralisation fluid [0.4% sodium thioglycolate in saline (STS)] to bind residual silver ions that may carry over from the reaction vessel and interfere with the quantification of viable cells. Serial dilutions were made and were plated onto Mueller–Hinton agar (MHA) to count viable cells and to calculate log reduction from the negative control inoculum. The 4-h log reduction of MRSA followed the same general procedure except 5 cm × 5 cm cut dressings were submerged in 10 mL of simulated wound fluid (SWF) (50:50 foetal calf serum : peptone water) inoculated to 1×10^6 CFU/mL from an overnight culture of the MRSA isolate (in triplicate). Following 4 h of incubation at 37 °C, a sample was removed and was diluted 1/10 in 0.4% STS, was serially diluted and was plated for viable cell counts. Log reduction was calculated by subtracting the viable cell counts after exposure to the dressing from the viable cell counts of the inoculum or in the case of longitudinal studies from the negative control vessel, and plotting the log transformed values.

2.4. Biofilm log reduction assay

Biofilms were grown on three to five layers of sterile cotton gauze and were placed in SWF in 6-well tissue culture plates for each strain tested. A 1×10^6 CFU/mL inoculum was added every 24 h up to 72 h and the plates were incubated at 37 °C with shaking at 200 rpm. After the incubation period, the gauze was removed from the liquid culture medium, was rinsed three times with sterile water and was placed onto the surface of a MHA plate (Oxoid, Nepean, Canada). The gauze biofilms were overlaid with additional MHA cooled to ca. 50 °C such that one-half of the biofilms were embedded in the agar and one-half were exposed. Dressings (5 cm × 5 cm) were overlaid onto the biofilms and were exposed at 37 °C for 4 h or 24 h. After the exposure time, the dressings and biofilms (gauze pieces) were carefully removed from the plates and were placed into 10 mL of 0.4% STS. They were vortexed (3 × 1 min) to disrupt the biofilm, were serially diluted in saline (0.9% saline) and were spot-plated onto MHA for viable cell counts. Negative controls were made as follows: (i) gauze biofilms were grown and were overlaid with agar as