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Antimicrobial activity of gallium maltolate against *Staphylococcus aureus* and methicillin-resistant *S. aureus* and *Staphylococcus pseudintermedius*: An *in vitro* study

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ABSTRACT

Gallium is a trivalent semi-metallic element that has shown antimicrobial activity against several important human pathogens. This antimicrobial activity is likely related to its substitution in important iron-dependent pathways of bacteria. The genus *Staphylococcus*, which includes human and animal pathogens that cause significant morbidity and mortality, requires iron for growth and colonization. In this study, gallium maltolate, at various concentrations between 50 and 200 μM, inhibited the *in vitro* growth of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) at time-points between 8 and 36 h after inoculation. The inhibitory activity of gallium maltolate against clinical isolates of MRSA and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) from a veterinary teaching hospital was determined.

1. Introduction

Gallium is a trivalent semi-metallic element that may have efficacy as a novel antimicrobial agent. Gallium has *in vitro* bactericidal activity against *Mycobacterium avis* (Olakanmi et al., 1997), *Pseudomonas aeruginosa* (DeLeon et al., 2009), *Rhodococcus equi* (Martens et al., 2007a,b), *Salmonella* Newport (Nerren et al., 2011), and *Staphylococcus* species (Baldoni et al., 2010). The antimicrobial effect of gallium relates to its biochemical similarity to iron. Because the ionic radii and other properties of Ga^{3+} and Fe^{3+} are very similar, Ga^{3+} can substitute for Fe^{3+} in iron-dependent biological processes such as bacterial iron scavenging and transport systems as well as enzyme synthesis pathways. In fact, Ga^{3+} is taken up preferentially over Fe^{3+} by some bacteria, including *P. aeruginosa* (Kaneko et al., 2007). GaM has shown efficacy against *P. aeruginosa* infection following burn injury in a murine model (DeLeon et al., 2009).

As most pathogenic bacteria are dependent upon iron (Ratledge and Dover, 2000; Rouault, 2004), the use of gallium may represent a new treatment strategy against bacterial infection. *Staphylococcus* species have iron requirements for important physiological pathways (Lindsay and Riley, 1994), and have become a significant zoological pathogen due to the morbidity and mortality associated with infection (Anwar et al., 2009; Weese, 2010). Although approximately 30% of the human population is colonized by *S. aureus* with no consequence, colonization can lead to serious local infection and or hematogenous spread

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(Lowy, 1998). *S. aureus* is a major cause of surgical site infection and hospital-acquired bacteremia, and of subsequent life-threatening infection manifesting as necrotizing fasciitis and pneumonia, septic arthritis, osteomyelitis, and endocarditis (Frazee et al., 2005a,b; Chambers and De Leo, 2009). Mortality rates from such infections are approximately 30%. Resistance to conventional antibiotic therapy has increased the virulence of this species (Weese, 2010), with methicillin-resistant *S. aureus* (MRSA) becoming epidemic in communities (Chambers and De Leo, 2009). Preliminary studies have indicated that GaM may be effective against the growth of *Staphylococcus* species *in vitro* (Baldoni et al., 2010).

The purpose of this research was to (1) investigate the growth of *S. aureus* and MRSA under varying concentrations of GaM *in vitro* and (2) determine the minimum inhibitory concentration (MIC) of GaM to clinical isolates of methicillin-resistant staphylococci from a veterinary patient population.

2. Materials and methods

2.1. Bacteria and growth conditions

Staphylococcus aureus subsp. *aureus* (ATCC 29213) and methicillin-resistant *S. aureus* subsp. *aureus* (ATCC 43300) were cultured in brain heart infusion broth (BHIB; Beckton, Dickinson and Company, Sparks, MD, USA) for 24 h at 37 °C on an orbital shaker at 250 rpm (Troemner Henry Analog Orbital Shaker OS-500, Northbrook, IL, USA). Bacterial cells were pelleted by centrifugation at $5000 \times g$ for 5 min and washed 3 times with sterile phosphate-buffered saline (PBS; Gibco BRL, Frederick, MD, USA). The concentration of bacteria was determined spectrophotometrically (Smartspec 3000; Bio-Rad Laboratories, Hercules, CA, USA) at an optical density of 600 nm, and approximately 5×10^6 colony forming units (CFU)/ml were inoculated into staphylococcal siderophore detection media (SSD) (Lindsay and Riley, 1994). SSD was used as the control medium, and SSD without added iron (SSD-Fe) was used to assess the effects of gallium on growth of *S. aureus* and methicillin-resistant *S. aureus*. All media were prepared in polypropylene beakers (VWR, Aurora, CO, USA) with molecular grade water (Milli-Qplus, 18X, pH 7.0; Millipore, Molsheirn, France), and sterilized through a 0.2-μM cellulose acetate filter into polystyrene containers (Nalgene polystyrene filter units, PES membrane, VWR). In all experiments, concentrations of bacteria were determined by 10-fold serial dilutions cultured in duplicate on brain heart infusion agar (Beckton, Dickinson and Company, Sparks, MD, USA). Bacterial concentrations were determined at 0, 8, 24, and 36 h and reported as CFU/ml. The experiment investigating the growth of *S. aureus* was replicated six times while the experiment involving methicillin-resistant *S. aureus* was conducted in triplicate.

2.2. Addition of gallium maltolate

Gallium maltolate (Chiral Quest Inc., Monmouth Junction, NJ, USA), was prepared as a 0.1 M sterile solution. The concentrations of *S. aureus* and methicillin-resistant *S. aureus* grown in SSD-Fe containing GaM at 50, 100, 150, and 200 μM were determined at 0, 8, 24, and 36 h; these were compared with the concentrations of *S. aureus* and MRSA grown in SSD. Results were quantified as colony forming units (CFU) per ml. Each experiment was carried out in duplicate.

2.3. GaM MIC of methicillin-resistant clinical isolates of Staphylococcus species

The MIC of GaM against each of 122 veterinary clinical isolates of methicillin-resistant *S. aureus* and *S. pseudintermedius* (MRSP) was determined. Clinical isolates were collected from 98 non-related patient specimens (97 dogs and 1 cat) and 24 samples obtained from routine environmental monitoring at the Veterinary Teaching Hospital, College of Veterinary Medicine and Biomedical Sciences (Texas A&M University) admitted between June 2006 and July 2010. Methicillin resistance was confirmed by two or more of the following tests: broth microdilution procedure, PCR for *mecA* gene, or oxacillin disk diffusion or agglutination test for PBP2A'. Thirty-four isolates were *S. aureus* and 88 were *S. pseudintermedius*. Gallium maltolate was suspended in RPMI 1640 media (GIBCO Invitrogen) at a concentration of 8 mg/ml. All RPMI 1640 media were supplemented with 5 ml sodium pyruvate (100 mM stock solution; GIBCO Invitrogen) and 5 ml glutamax solution (200 mM stock solution; GIBCO Invitrogen) per 500 ml of RPMI. *Staphylococcus* isolates were plated onto Columbia agar plates supplemented with 5% sheep's blood. Isolated colonies were suspended in RPMI 1640 media at a concentration equivalent to a McFarland 0.5 standard. A 50 μl aliquot of this standard was inoculated into 5 ml of RPMI 1640 media. All MIC tests were performed using 96-well plates. All dilutions were 2-fold dilutions starting at 4 mg/ml gallium maltolate and ending at 0.0625 mg/ml. Wells containing RPMI medium with or without the *Staphylococcus* isolate were included as positive and negative control wells, respectively. Each 96-well plate included 6 test isolates. *S. aureus* ATCC 43300 and *S. aureus* ATCC 29213 were included on every 96-well plate as methicillin resistant and susceptible controls to ensure consistency of testing throughout. Gallium maltolate MICs for *S. aureus* ATCC 43300 and *S. aureus* ATCC 29213 were consistent with those reported previously (Baldoni et al., 2010).

The MIC was defined as the lowest GaM concentration that prevented visible bacterial growth.

2.4. Statistical analysis

The data from the growth of *S. aureus* and MRSA with varying concentrations of GaM were log-transformed to meet the distributional assumptions of the linear mixed-effects models. The log CFU count was the dependent variable, and the independent variables were treatment, time, and their interactions. Individual experiments were modeled as a random effect to account for repeated measures within the experiment. Time was considered an ordered category to facilitate comparisons within and among times. Post hoc testing of treatment effects within