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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.

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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

Table 1
The log CFU average of 6 experiments investigating the growth of *Staphylococcus aureus* subspecies *aureus* with various concentrations of gallium maltolate.

Treatment	Time (h)			
	0	8	24	36
Control	6.2 (5.7–6.7)^{a,1}	8.4 (7.8–9.0)^{b,1}	8.7 (8.1–9.3)^{b,1}	8.0 (7.4–8.6)^{b,1}
Control no Fe	6.4 (5.8–7.0)^{a,1}	7.8 (7.0–8.7)^{b,1}	8.2 (7.3–9.0)^{b,1}	7.6 (6.8–8.4)^{b,1}
+GaM50	6.2 (5.6–6.8)^{a,1}	7.9 (7.1–8.8)^{b,1}	7.4 (6.6–8.3)^{b,2}	7.3 (6.4–8.1)^{b,1}
+GaM100	6.2 (5.6–6.8)^{a,1}	7.9 (7.1–8.8)^{b,c,1}	7.1 (6.3–8.0)^{a,c,2}	6.9 (6.1–7.8)^{a,c,2}
+GaM150	6.3 (5.7–6.9)^{a,1}	7.9 (7.0–8.9)^{b,c,1}	6.9 (6.1–7.8)^{a,c,2}	6.5 (5.6–7.3)^{a,2}
+GaM200	6.3 (5.7–6.9)^{a,1}	7.6 (6.8–8.5)^{b,c,1}	7.0 (6.1–7.8)^{a,c,2}	6.4 (5.5–7.2)^{a,2}

Values with different letters differ significantly ($P < 0.05$) within row.
Values with different numbers differ significantly ($P < 0.05$) within column.

Table 2
Log CFU average of 3 experiments investigating the growth of methicillin-resistant *Staphylococcus aureus* subspecies *aureus* with various concentrations of gallium maltolate.

Treatment	Time (h)			
	0	8	24	36
Control	14.0 (13.6–14.4)^{a,1}	19.3 (18.8–19.9)^{b,1}	18.4 (17.8–19.0)^{b,1}	17.8 (17.2–18.3)^{b,1}
Control no Fe	14.0 (13.6–14.4)^{a,1}	18.5 (17.7–19.3)^{b,1}	17.0 (16.2–17.8)^{b,1}	14.2 (13.4–15.0)^{a,2}
GaM50	14.0 (13.6–14.4)^{a,1}	18.5 (17.7–19.3)^{b,1,2}	14.0 (13.2–14.8)^{a,2}	12.8 (12.0–13.6)^{a,2}
GaM100	14.0 (13.6–14.4)^{a,1}	17.1 (16.3–17.9)^{b,2,3}	13.1 (12.3–13.9)^{a,2}	13.0 (12.5–13.8)^{a,2}
GaM150	14.0 (13.6–14.4)^{a,1}	16.2 (15.4–17.0)^{b,3,4}	13.5 (12.7–14.3)^{a,2}	13.0 (12.2–13.8)^{a,2}
GaM200	14.0 (13.6–14.4)^{a,1}	15.4 (14.6–16.2)^{b,4}	13.8 (13.0–14.6)^{a,2}	13.4 (12.6–14.2)^{a,2}

Values with different letters differ significantly ($P < 0.05$) within row.
Values with different numbers differ significantly ($P < 0.05$) within column.

time and time effects within treatment were made using the method of Scheffé. A P value of <0.05 was considered significant.

The MIC of GaM to clinical strains of MRSA and MRSP was recorded. The MIC values to MRSA and MRSP were compared using a Wilcoxon rank-sum test.

3. Results

The results of the inhibition of laboratory strains of *S. aureus* and MRSA by GaM are listed in Tables 1 and 2. The MICs of GaM to clinical isolates of methicillin-resistant *S. aureus* and *S. pseudintermedius* are listed in Table 3 and Figs. 1 and 2.

Results of the 6 experiments of *S. aureus* grown under varying concentrations of gallium maltolate revealed significant effects of time and time-by-treatment interaction.

Considering the effects of time accounting for treatment, there was a significant increase after 8, 24, and 36 h in the CFU for both controls (Table 1). The same was true for the GaM 50 μM dose, although the increase tended to be lower for GaM 50 μM than for controls. For the other GaM doses (100, 150, and 200 μM), CFUs were significantly ($P < 0.05$) greater than baseline after 8 h of growth, but were not significantly different than baseline after 24 and 36 h.

Considering the effects of treatment accounting for time (Table 1), there were no significant differences among treatments at time 0. Although the CFU counts for the control without iron tended to be higher than those for the control with iron, there was no significant difference between the 2 control groups at any time. After 8 h, there were no significant differences among any treatments. After 24 h, GaM 50 μM ($P = 0.0028$), GaM 100 μM ($P = 0.0004$), GaM 150 μM ($P < 0.0001$), and GaM 200 μM ($P < 0.0001$) had significantly lower CFUs than the control with iron; they all also had significantly ($P < 0.05$) lower growth than the control with no iron. Although the data suggest a dose-response effect, there were no significant differences among GaM doses at 24 h. After 36 h, although the CFU tended to be lower for GaM 50 μM dose, the difference was not significantly different from either control. The CFUs were significantly lower than the control with iron after 36 h for GaM 100 μM ($P = 0.0210$), GaM 150 μM ($P = 0.0004$), and GaM 200 μM ($P = 0.0001$); these 3 also were significantly ($P < 0.05$) lower than the control with no iron.

For the experiments investigating the effects of the growth of MRSA with varying concentrations of GaM, there were significant effects of time, treatment, and the time-by-treatment interaction. Considering the effects of time within treatment (Table 2), the control values were significantly higher at 8, 24, and 36 h than at baseline; however, there

Table 3
The inhibition of clinical isolates of methicillin-resistant *Staphyococcal* species by GaM.

Species	Concentration GaM (mg/ml)								Total
	<0.125	0.125	0.25	0.5	1.0	2.0	4.0	>4.0	
MRSA	1	2	11	2	3	3	3	9	34
MRSP	0	0	1	0	3	4	2	78	88

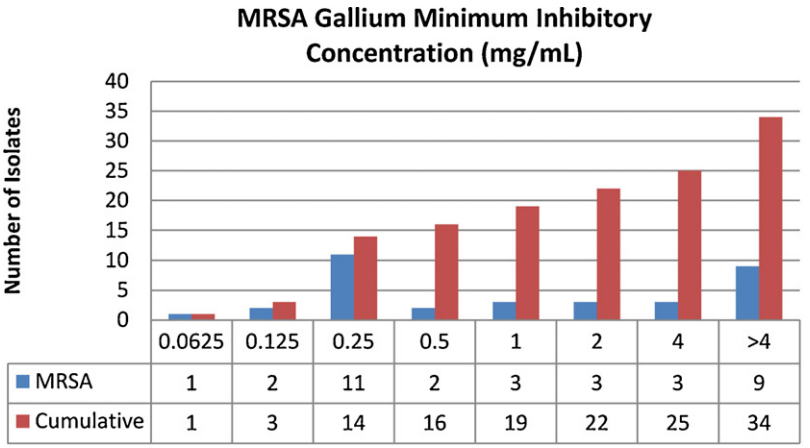

Fig. 1. The MIC of GaM against isolates of MRSA.

were no significant differences between any other pairs of times. The control without iron was significantly different from time 0 h compared to the 8 and 24 h time-points, but not at 36 h. The 8-h sample was not significantly lower than that at 24 h. The 8-h sample and 24-h sample were significantly higher than the 36-h sample. The CFU values at GaM concentrations of 50, 100, 150, and 200 μM were significantly greater at 8 h than at 0, 24, and 36 h, but no other times were significantly different.

Considering the effects of treatment within time, all CFUs were the same among the various treatments at 0 h. At the 8-h time point, the CFU values for both controls and the 50 μM GaM dose were significantly higher than for all other doses. There were significantly different values between the other doses at this time point. By 24 h, all concentrations of GaM yielded significantly lower CFU/ml than either control, and the controls did not differ significantly (although the control with no Fe tended to be lower). There were no significant differences among the GaM treatments detected at 24 h. At 36 h, the control with iron yielded significantly greater CFUs than all other treatments. Although the CFU values for the controls with no iron tended to be higher than those for the GaM treatments, the differences were not significant.

The minimum inhibitory concentration of GaM was determined for 122 methicillin resistant veterinary staphylococcal isolates (Table 3). Of these, 34 were *S. aureus* while 88 were *S. pseudintermedius*. Over 50% of veterinary *S. aureus* isolates had a MIC < 1 mg/μl GaM (Fig. 1). In contrast, only 5% of MRSP isolates had an MIC of <1 mg/μl GaM (Fig. 2). The MIC values for GaM against MRSP (median, >4 mg/ml; range 0.25 to >4 mg/ml) were significantly ($P < 0.0001$) greater than those for MRSA (median, 1.0 mg/ml; range 0.0625 to >4 mg/ml).

4. Discussion

GaM has potential to serve as a novel antimicrobial agent. Unlike conventional antibiotic therapies, GaM has a unique mechanism of action, and therefore does not face traditional issues associated with antimicrobial resistance. GaM exploits the needs of pathogenic bacteria for iron in important metabolic and enzymatic pathways; it can thus inhibit DNA and protein synthesis and redox reactions. Gallium is preferentially concentrated in inflamed tissues,

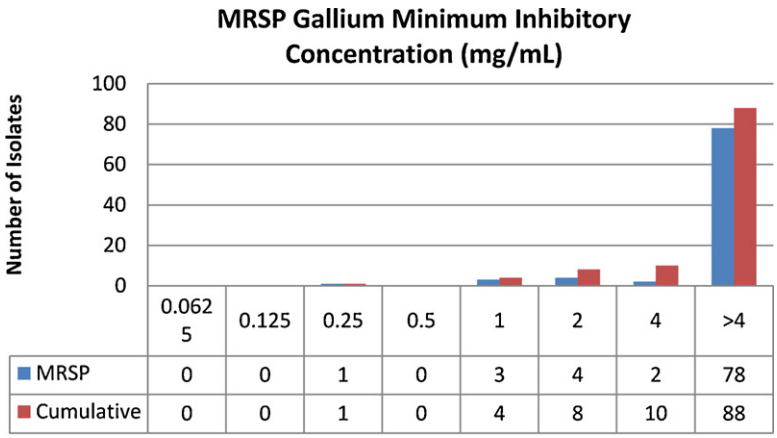

Fig. 2. The MIC of GaM against clinical isolates of MRSA.

macrophages, neutrophils and bacteria (Bernstein, 1998). GaM has good bioavailability in humans, and has been used safely in other mammals (Bernstein, 2005). Its use is not associated with tissue toxicity and it can be given orally without side-effects. These characteristics make it an ideal candidate for further study as an antimicrobial agent.

GaM has been proven to have *in vitro* activity against important human pathogens such as *M. avis* (Olakanmi et al., 1997), *P. aeruginosa* (DeLeon et al., 2009), *R. equi* (Martens et al., 2007a,b), *Salmonella* Newport (Nerren et al., 2011) and staphylococci (Baldoni et al., 2010). In particular, its ability to inhibit growth of staphylococci may be of great interest as this pathogen is now a significant source of human and veterinary morbidity and mortality. The results of this study showed that GaM at doses of 50–200 μM inhibited the *in vitro* growth of *S. aureus* and MRSA at between 8 and 36 h by 1 or 2 logs (i.e., 90% of exposure. These results were statistically significant, representing a one to 99% reduction in growth).

The MIC values of GaM to veterinary clinical isolates of staphylococci in this study were comparable to those for human isolates (Baldoni et al., 2010). Baldoni et al. (2010) examined isolates of *S. aureus* and *S. epidermidis* that were sensitive or resistant to methicillin. In our veterinary hospital, methicillin resistant *S. aureus* (MRSA) and *S. pseudintermedius* (MRSP) are frequently encountered. Over 50% of our MRSA isolates had a MIC of <1 mg/ml GaM, similar to the results reported by Baldoni et al. (2010). In contrast, only 5% of our MRSP isolates had an MIC of <1 mg/ml GaM, whereas 89% of MRSP isolates had an MIC of >4 mg/ml. The MIC of GaM to *S. epidermidis*, including those resistant to methicillin, was much lower, at <0.2 mg/ml (Baldoni et al., 2010). While the extent and mechanisms of variations in MIC values among staphylococcal species are unknown, the results of the two studies indicate that GaM might have potential use as an antimicrobial agent for staphylococci.

Given that GaM may be bactericidal against important pathogenic bacteria *in vitro*, the question remains as to whether it is effective *in vivo*. Recent clinical studies investigating its use in the prevention of *R. equi* pneumonia in foals (Chaffin et al., 2011) and the reduction in fecal shedding of *Salmonella* in cattle (Nerren et al., 2011) have been disappointing. A proposed mechanism for the discrepancy between bactericidal activity *in vitro* and *in vivo* is the low serum concentrations achieved after systemic administration. In contrast, DeLeon et al. (2009) found the compound effective for preventing and treating local wound infections in a murine model of burn wound infection. There is much unknown about the serum concentration of GaM needed for therapeutic effect in veterinary species. Based upon limited work in the horse (Chaffin et al., 2010, 2011; Arnold et al., 2010), either increased systemic dosages are needed to provide bactericidal activity, or therapeutic potential should be directed towards topical or local treatment. The results of this study indicate that GaM may be useful in the treatment of *Staphylococcal* infections in veterinary species infected with *S. aureus*, particularly when used as a topical or local therapy.

5. Conclusion

Concentrations of GaM between 50 and 200 μM were found to inhibit the growth of *S. aureus* and MRSA *in vitro* at 24 and 36 h; there were no significant differences between concentrations. Concentrations of GaM between 100 and 200 μM were also found to inhibit the growth of MRSA *in vitro* at 8 h. The MICs for MRSP were higher than those of MRSA in veterinary clinical isolates. These results support the continued investigation of GaM as a potential antimicrobial agent for staphylococcal infections in animals.

Conflict of interest

The authors have no conflicts of interest.

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