Evaluation of gallium citrate formulations against a multidrug-resistant strain of 
*Klebsiella pneumoniae* in a murine wound model of infection

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Skin and soft tissue infections (SSTIs) are a common occurrence in healthcare facilities with a heightened risk for immunocompromised patients. *Klebsiella pneumoniae* has been increasingly implicated as the bacterial agent responsible for SSTIs, and treatment can be challenging as more strains become multidrug-resistant (MDR). Therefore, new treatments are needed to counter this bacterial pathogen. Gallium complexes exhibit antimicrobial activity and are currently being evaluated as potential treatment for bacterial infections. In this study, we tested a topical formulation containing gallium citrate (GaCi), for the treatment of wounds infected with *K. pneumoniae*. First, the minimal inhibitory concentration against *K. pneumoniae* ranged from 0.125 to 2.0 ug/mL GaCi. After this *in vitro* efficacy was established, two topical formulations with GaCi (0.1% w/v and 0.3% w/v) were tested in a murine wound model of MDR-*K. pneumoniae* infection. Gross pathology and histopathology revealed *K. pneumoniae*-infected wounds appeared to close faster with GaCi treatment and was accompanied by reduced inflammation when compared to untreated controls. Similarly, quantitative indications of infection remediation such as reduced weight loss and wound area suggested that treatment improved outcome when compared to untreated controls. Bacterial burdens were measured one and three days following inoculation, and a 0.5 – 1.5 log reduction of colony forming units was observed. Lastly, upon scanning electron microscopy analysis, GaCi treatment appeared to prevent biofilm formation on dressings when compared to untreated controls. These results suggest that with more preclinical testing a topical application of GaCi could be a promising alternative treatment strategy for *K. pneumoniae* SSTI.
The demand for novel antibacterial treatments is growing given that many species and strains of bacteria worldwide have become increasingly resistant to the majority of clinically available antibiotic treatments. Specifically, *Klebsiella pneumoniae*, a member of the ESKAPE pathogens (*Enterobacter* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterococcus* spp.) has been responsible for numerous outbreaks in a number of hospitals (1-4), including a recent, fatal outbreak at the National Institutes of Health in the United States (5). *K. pneumoniae* isolates were also the first bacterial species shown to harbor the NDM-1 plasmid, a plasmid that harbors the New Delhi metallo-beta-lactamase (NDM-1) responsible for extensive carbapenem resistance (6). Increases in the number of *K. pneumoniae* infections have been observed in U.S. military hospitals with the shift to the conflict in Afghanistan as compared to *A. baumannii* infections that were more prevalent during Operation Iraqi Freedom (7). Moreover, colistin-resistant *K. pneumoniae* strains have started to emerge around the world (8-10) suggesting that this bacterial species can evolve quickly even in the face of aggressive antibiotic treatments. The combination of nosocomial spread with multidrug-resistant (MDR) *K. pneumoniae* strains is a daunting prospect for clinicians as there are few treatment options remaining on the foreseeable horizon.

Iron sequestration or competition for iron has been explored for many years as a potential therapy for bacterial infection. In fact, the human body utilizes the iron sequestration approach by limiting free iron at infection sites with molecules such as lactoferrin, transferrin, hepcidin, and other iron-binding proteins in order to limit bacterial growth (11-13). However, bacterial pathogens have evolved a number of mechanisms to
counter these host defenses by capturing iron from various sources. For example, the iron acquisition systems such as the Feo, Sit, and Efe transport free ferrous iron, Fe(II), into the bacterial cell in Gram negatives (13, 14) and are an essential process for many bacterial species (15-18). FeoB is well-conserved across Gram negatives and was shown to localize in the inner, cytoplasmic membrane, where Fe(II) transport appears to be dependent on GTP activity (19). The Sit acquisition system appears to be specialized for intracellular, enteric bacteria such as *Shigella* or *Salmonella* species, where Fe(II) is depleted in the cytoplasm of the host (17, 20). In contrast, Enterohaemorrhagic *E. coli* does not invade cells, but presence of the Efe system helps to transport Fe(II) inside the bacteria and confer a survival advantage in the low pH environment of the gastrointestinal tract (21).

However, bacteria also utilize ferric iron, Fe(III), for survival and employ siderophores, small molecules that are secreted into the extracellular milieu in order to capture Fe(III) from the host (11-13). There are a large variety of siderophores with a high binding affinity for Fe (III), and their cognate receptors are found on the surface of the outer membrane (11-13). Siderophores are often unique to each bacterial species, and are required for survival in the host environment (12, 18). Lastly, bacteria have been shown to harbor membrane-bound receptors for the human proteins transferrin, lactoferrin, and hemin to traffic the iron that is complexed by these host proteins into the infecting bacterium. (11-13). All of these iron acquisition mechanisms speak to the significant importance of iron for bacterial survival in the host and were likely derived from systems used by progenitors that were fighting for iron and other nutrients in restricted, non-host environments such as the soil.
In an attempt to counter iron-dependent bacterial processes, researchers have attempted treatment with iron chelators with high binding constants to outcompete the siderophores and other bacterial iron acquisition mechanisms (22-25). However, the use of iron chelators as an antimicrobial therapeutic approach has had mixed success and is likely reflective of the chelator choice and the indication pursued (26). Another promising antibacterial method that exploits the requirement for iron is a “Trojan horse” strategy, where siderophores are conjugated to antibiotics (sideromycins) via linkage chemistry (27, 28). This antibacterial approach has had successes both in vitro and in vivo (29-33); however, resistance could also rapidly emerge because many bacteria harbor multiple siderophore receptors, and there are also “cheaters”, bacteria that usurp siderophores other than their own, which are prevalent in polymicrobial infections (34-39). A recent failure of a sideromycin in vivo also points to efflux as a mechanism of resistance (33); however, a follow-up study showed efflux could be overcome with the application of an efflux inhibitor (40).

Another approach exploiting the bacterial need for iron could be the use of simple gallium (III) salts such as gallium nitrate, gallium maltolate, or gallium citrate (Fig. 1). In this case, rather than chelating iron away from the bacterial pathogen, gallium, in excess, outcompetes the iron that is targeted by the aforementioned acquisition systems. Then, upon uptake, it replaces the iron in key chemical reactions required for survival and ultimately poisons the bacteria (41). This approach has shown efficacy against P. aeruginosa both in vitro and in vivo (42-46), and treatment success may also involve anti-biofilm activity (44-46). Gallium formulations have also been shown to be effective against other pathogenic bacteria (47-51) to include A. baumannii (52, 53); however, it should be
noted that certain strains of *A. baumannii* appear to circumvent gallium nitrate activity with a heme receptor in the host environment (54). Given this mixed efficacy against different bacteria, each gallium formulation needs to be thoroughly tested against each pathogen both *in vitro* and *in vivo*. Moreover, there is also the challenge of toxicity. Gallium (III) has been shown to be safe in lower doses, where specifically, gallium citrate has been used as a diagnostic for cancer and gallium nitrate has been approved for human use as a cancer treatment (Ganite®) (41, 55, 56). However, any formulation at a higher concentration has the potential to limit cell division (i.e. 100 µM for human keratinocytes) or cause apoptosis (i.e. 500 µM for human keratinocytes) (56).

In the current study, we explore the use of gallium citrate (GaCi) against *K. pneumoniae*. Like the other pathogenic bacterial species previously mentioned, *K. pneumoniae* isolates have a high demand for iron, especially in the host environment, and recently, a hypervirulent strain was shown to have an increased propensity for iron acquisition (57). As *K. pneumoniae* has been responsible for many wound infections suffered by the U.S. military (7, 58) as well as chronic wounds suffered by diabetics (59), a topical therapy to combat skin and soft tissue infections (SSTIs) caused by this pathogen would be desirable.
MATERIALS AND METHODS

Bacterial strains and inocula preparation.

The majority of *K. pneumoniae* isolates tested were wound infection clinical isolates from International Health Management Associates, Inc. except for BAA-2146™, which was obtained from the American Type Culture Collection (ATCC®) and KP4640, which was obtained from the Multidrug-resistant Organism Repository & Surveillance Network (MRSN) at the Walter Reed Army Institute of Research (WRAIR). KP4640 was previously described (60), and this isolate was used for all of the in vivo wound infection experiments in this study. KP4640, before use in animals, was propagated on Lennox lysogeny broth (LB) (Becton, Dickinson and Co., Sparks, MD). To prepare inocula for animal infection, 100 µL of a KP4640 overnight culture was subcultured into 10 mL of fresh LB in a 250 mL Erlenmeyer flask, and grown at 37°C and shaking at 250 rpm. Cells in mid-exponential growth phase were harvested at an OD₆₀₀ of 0.8. Cells were washed twice with sterile phosphate buffered saline (PBS) and then resuspended in PBS so that 25 µL of the suspension contained approximately 5.0 x 10⁵ cells. The cell concentration of the suspension was verified via a Petroff-Hauser counting chamber prior to inoculation of mice, and confirmed by serial dilution and plating on LB agar using a spiral plating system (Autoplate®, Advanced Instruments, Inc., Norwood, MA). The difference observed between each measure of cell number was never more than two-fold.

Minimal Inhibitory Concentration

The minimal inhibitory concentration (MIC) was determined by International Health Management Associates, Inc. or Aridis Pharmaceuticals, Inc. using an overnight culture of...
bacteria that was diluted to a starting inoculum of ~2.0 x 10^5 CFU/mL and assessing
turbidity after 24 hours at 37°C and exposure to GaCi serially diluted using two-fold
dilutions with a range from 0.02 – 256 µg/mL. To mimic the in vivo, low iron
concentration environs of the host, an iron-deficient culture media (BM-2) with succinic
acid as a sole carbon source (40 mM K_2HPO_4, 22 mM KH_2PO_4, 7 mM (NH_4)_2SO_4, 34 mM succinic
acid, and 1 mM MgSO_4 adjusted to pH 7.0) was utilized for all of the MIC experiments instead of the
standard cation-adjusted Mueller-Hinton broth, which is iron-rich and typically used.
Separately, an antibiogram for KP4640 was determined using the Phoenix (Becton,
Dickinson and Co., Franklin Lakes, NJ) automated system according to the manufacturer’s
instructions.

**Preparation of treatments**

The neutropenic agent cyclophosphamide was purchased in powdered form from
Baxter (Deerfield, IL), and dissolved in sterile 0.9% sodium chloride injection solution
(Hospira Inc., Lake Forest, IL) to a final concentration of 10 mg/mL. Antibiotics, either
colistin (Sigma-Aldrich), doxycycline (Sigma-Aldrich) or Primaxin™ (Baxter) were
purchased in powdered form and dissolved in dissolved in sterile 0.9% sodium chloride
injection solution (Hospira Inc., Lake Forest, IL) and, if needed, sterile dimethyl sulfoxide
(DMSO) was used to improve solubility and achieve final concentrations of 10 mg/mL.
Placebo hydroxyethyl cellulose (HEC) formulations as well as HEC amended with either
0.1% w/v or 0.3% w/v GaCi were provided by Aridis Pharmaceuticals, Inc. Gallium citrate
was prepared by mixing gallium nitrate with ammonium citrate, followed by precipitation
of gallium citrate and removal of nitrate based previously known chemical reactions (56). This manufacturing process has been implemented at kilogram scale under GMP (Good Manufacturing Practice).

Mice and husbandry
Female BALB/c mice were purchased from the National Cancer Institute (now Charles River), Animal Production Program (Frederick, MD). The mice used in these experiments were six to ten weeks of age and weighed 14 to 20 g. All mice received sterile food and water ad libitum, and dry rodent chow was supplemented with DietGel® Recovery (ClearH2O®, Portland, ME) during the 48 h following wounding. All mice were housed in groups of three, in sanitized cages on sterile paper bedding, and were provided with environmental enrichment, including in-cage plastic housing.

Murine dorsal wound model
All procedures were performed in accordance with protocol IB02-10 or 14-BRD-01S that was approved by the Walter Reed Army Institute of Research (WRAIR)/Navy Medical Research Center (NMRC) Institutional Animal Care and Use Committee (Silver Spring, MD) and were previously described as a model for A. baumannii wound infection (61). Briefly, mice received 150 mg/kg and 100 mg/kg cyclophosphamide intraperitoneal (IP) injections, before wounding and infection, on days -4 and -1, respectively. On day 0, the day of wounding and inoculation, mice were anesthetized with ketamine 130 mg/kg (Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA), xylazine 10 mg/kg (AnaSed®, Lloyd Inc. Shenandoah, IA), and buprenorphine 0.05 mg/kg (Hospira Inc., Lake Forest, IL) injections
were given for pain management. Hair was clipped from the cervical to mid-lumbar
dorsum, and the skin scrubbed with iodine solution followed by an ethanol rinse. A 6.0 mm
disposable skin biopsy punch (VisiPunch, Huot Instruments, LLC, Menomonee Falls, WI)
was used to create a full-thickness skin defect overlying the thoracic spinal column and the
adjacent musculature. Twenty-five µL containing 5.0 x 10^5 KP4640 cells in a PBS
suspension were pipetted onto the wound and allowed to absorb for at least three minutes.
A circular cutout (30 mm diameter) of transparent dressing (Tegaderm™ Roll, 3M Health
Care, St. Paul, MN) was placed over the wound and secured with tissue adhesive (Vetbond™,
3M Animal Care, St. Paul, MN).

For experiments assessing weight loss and wound closure, beginning at four hours
(4 hr) post-inoculation, mice (3 biological replicates with 12 mice per group, a total of 144
mice) were treated with either 25 µL of HEC placebo, 0.1% w/v GaCi, or 0.3% w/v GaCi.
Subsequently and for the next three days, infected wounds were treated either once-daily
(OD) with 0.3% w/v GaCi, twice-daily (BID) with 0.1% w/v GaCi, or 25 µL of HEC placebo.
On day 3, after the last treatment, the transparent dressing was removed, treatment was
discontinued, and the wound was monitored for closure through day 15 and sometimes to
day 20.

In experiments investigating wound CFU burden, mice (3 biological replicates with
6 mice per group) were treated at 4 hr post-inoculation, and subsequently, treated with
either placebo HEC (BID) 0.1% GaCi w/v BID, or 0.3% GaCi w/v OD in HEC. CFU
enumeration data was pooled from all three experiments.

Separately, another set of mice (2 biological replicates of 5 mice per group, 10 mice
total) beginning at 4 hr post-inoculation, were treated topically with either 25 µL of sterile
saline, 25 μL of colistin at a final concentration of 2.5 mg/kg applied topically, Primaxin™ 2.5 mg/kg via intraperitoneal (IP) injection, or doxycycline at 25 mg/kg via IP injection. Subsequently, mice were dosed BID with these preparations every day for three days thereafter as previously described above.

Quantitative and qualitative wound closure assessments
Wound area measurements were taken on the day of wounding and at subsequent time points using a Silhouette™ wound measurement device (Aranz Medical Limited, Christchurch, New Zealand). Time course wound photographs assessing gross pathology were taken using a five megapixel iSight™ camera (Apple Inc., Cupertino, CA).

CFU Enumeration
To examine CFU burden within the wound bed, mice were euthanized with ketamine (250 mg/kg) and xylazine (25 mg/kg) overdose according to protocol on day 1 or day 3. A 4-mm disposable skin biopsy punch (Acuderm Inc., Fort Lauderdale, FL) was then used to sample a disc from the wound bed. The sample was manually disrupted in PBS and serial 10-fold dilutions were plated via a spiral plater (Autoplate; Advanced Instruments, Inc., Norwood, MA) on eosin methylene blue agar (Becton, Dickinson and Co., Sparks, MD). Plates were incubated overnight at 37°C, and then enumerated.

Separately, the CFU was determined at the 4 hr post-inoculation time point. The dorsal wounds were removed en bloc by severing the cervical and lumbar spinal column and trimming the tissue >2 cm beyond the wound edge. The tissue was processed using a
sterile homogenizer, and serial 10-fold dilutions were plated via a spiral plater and enumerated as above.

**Scanning electron microscope evaluation of wound bed and dressing biofilm**

Dressings and wound bed tissue were evaluated by scanning electron microscopy (SEM). A representative mouse from three separate experiments in the placebo, GaCi 0.1% BID and GaCi 0.3% OD treatment groups was sacrificed at four hours post-wounding and on day 7. The transparent dressings and a 4 mm tissue disc for each animal were fixed in 4% formaldehyde, 1% glutaraldehyde, 0.1 M PBS. The samples were washed three times using 0.1 M PBS, and then post-fixed in 1% osmium tetroxide in 0.1 M PBS for one hour. The samples were dehydrated in a graded series of ethanol solutions, and then dried (Critical point dryer, Model 28000, Ladd Research Industries, Burlington, VT). The samples were mounted by double-sided carbon tape to specimen stubs, and ion coated with gold:palladium (30:70) (Hummer X sputter coater, Anatech Ltd., Alexandria, VA). The samples were visualized using an Amray 3600 FE scanning electron microscope (Bedford, MA) operated at a voltage of 3 KV. Samples were analyzed by scanning ten or more 1000x magnified fields within the wounded tissue and on the portion of the dressing overlying the wounded area. Photomicrographs representative of the observed biofilm density were taken at 2500x magnification.

**Histological examination of the wound bed**

Mice from each treatment group (HEC OD, HEC BID, GaCi 0.1% BID, and GaCi 0.3% OD) were sacrificed on day 15 in order to characterize wound histopathology. The dorsal
wounds were removed en bloc by severing the cervical and lumbar spinal column and trimming the tissue >2 cm beyond the wound edge. The tissue was immediately fixed in phosphate-buffered formalin (10%) for >72 h. The wound tissue, consisting of spinal column and surrounding soft tissues, was then demineralized for 24 hours using Decal Stat™ (Decal Chemical Corp, Tallman, NY), rinsed with water for 3-5 minutes, and trimmed in a dorsal-ventral plane bisecting the spinal column and placed back into 10% phosphate buffered formalin. The wound tissue specimens were embedded in paraffin, cut in a dorsal-ventral plane bisecting the spinal column, mounted on positively charged glass slides (Colormark Plus, Thermo Scientific, Portsmouth, NH), and stained with hematoxylin (Astral Diagnostics, Inc., West Deptford, NJ) and eosin (Astral Diagnostics, Inc., West Deptford, NJ) for light microscopic examination.

Wounds were histologically assessed for presence and dissemination of bacteria, host immune response, indications of wound healing i.e., extent of epithelial migration, coverage, maturation, and amount of granulation tissue present within the wound and if wound and associated inflammation extended into underlying vertebrae, spinal canal, and/or spinal cord.

**Statistical analyses**

All statistical analyses were carried out using GraphPad Prism software. Wound sizes, CFU burdens and weight changes were compared via the Mann-Whitney U-Test, with Bonferroni corrections applied when necessary. All results were considered significant if $P<0.05$.
RESULTS

Minimal Inhibitory Concentrations

The MIC of GaCi was determined for numerous bacterial species (Table S1). Upon analysis of this data, *K. pneumoniae* was recognized as being more susceptible than most other bacterial species to GaCi. Therefore, the MIC of 15 *K. pneumoniae* clinical isolates from wound infections producing extended-spectrum beta-lactamase (ESBL) (Table 1). Also, the MIC of GaCi was determined for two *K. pneumoniae* isolates of clinical importance: the original NDM-1 isolate (ATCC® BAA-2146™) (6) and KP4640, which is a *Klebsiella pneumoniae* carbapenemase (KPC) isolate from a Wounded Warrior that has been previously described (60) and was also used for the subsequent animal experiments in this study (Table 1). The antimicrobial potency of GaCi was also compared to two antibiotics, tobramycin and aztreonam, which are still often used for treatment. Gallium treatment exhibited significantly lower MIC values than either the tobramycin (approximately 8-fold lower) or aztreonam (approximately 16-fold lower) (Table 1). To ensure that the different topical formulations that were to be used for application in murine model did not impact the MIC, the activity of GaCi formulated in HEC was also tested and shown to be identical to the unformulated results found in Table 1 (data not shown).

Time course gross pathology and wound size of treated and untreated wound infections

The first assessment of the murine wound model included a gross pathology comparison of wounds that were infected with *K. pneumoniae* and received GaCi-treatment versus no treatment. Initially, mice were treated on day 0 at four hours post-inoculation because at this time point, we saw almost a $2\log_{10}$ increase bacterial burden (Fig. S1) suggesting the bacteria were not just colonizing the wound but establishing infection. Subsequently, and
each day after for three days, wounds received either 0.1% GaCi twice-daily (BID) or 0.3% GaCi once-daily (OD) or HEC vehicle control. Photographs of the dorsal, full-thickness wounds were taken on days 7 and 15 (Fig. 1). On day 7, serocellular crust formation can be seen in all treatment groups. In BID (Fig. 1A) and OD (Fig. 1C) placebo-treated groups, swelling and redness is more pronounced around the wound compared to mice treated with 0.1% GaCi BID (Fig. 1B), or mice treated with 0.3% GaCi OD (Fig. 1D). By day 15 (Fig. 1E-H) wounds in all treatment groups displayed wound edge rounding, indicative of contracture. Separately, photographs were also taken on Day 20 with two sets of mice. The healing trends previously observed continued and wounds appeared to be closed with GaCi treatment in contrast to HEC vehicle-treated wounds that remained infected and open (Fig S2).

The wound area was measured on days 0, 6, 9, and 15, post-infection according to the Methods. When wounds were measured on day 6 post-infection, the HEC twice-daily group had median wound sizes of 61 mm² while 0.1% GaCi-treated had median wound sizes of 38 mm² (P<0.05) (Fig. 2). The same trends continued and by day 9, the HEC placebo-treated mice had a median wound size of 85 mm², while the mice treated with 0.1% GaCi had a median wound size of 54 mm² (P<0.05) (Fig. 2). By day 15 post-infection, groups treated with HEC BID had median wound sizes of 105 mm² while 0.1% GaCi-treated mice had a median wound size of 61 mm² (P<0.05).

Separately, we tested 0.3% GaCi treatments, and wounds were again measured via Aranz on days 0, 6, 9, and 15, post-infection. On day 6 post-infection, the once-daily gallium 0.3%-treated had a median wound size of 29 mm² while the HEC once-daily group had a median wound size of 24.5 mm², which was not statistically significant (P=0.856).
However, on day 9, the mice treated with the 0.3% GaCi formulation had a median wound size of 33 mm², which was significantly smaller than mice treated once-daily with the HEC control formulation that had a median wound size of 59 mm² ($P<0.0001$) (Fig. 3). By day 15 post-infection, groups treated with HEC once daily had a median wound size of 74 mm², but and mice treated with 0.3% GaCi had a median wound size of 34.5 mm² ($P<0.001$). Wound sizes between both experiments groups were not statistically different on day 0 when compared via Mann-Whitney Tests (Fig. 2 and Fig. 3).

Weight loss

Mice treated twice-daily with HEC lost a median of 10.3% of their infection-day body weight one day post-infection, 18.52% on day 2, and lost a median of 19.85% three days post-infection, while mice treated twice-daily with 0.1% w/v GaCi lost 13.11%, 18.52%, and 19.04% on those days respectively (Fig. S3). There was no significant difference between twice daily treated groups.

In contrast, mice treated with once-daily GaCi 0.3% w/v lost significantly less weight ($P<0.01$) when compared to the HEC once-daily control on day 2 post-infection. Specifically, mice treated once-daily with 0.3% w/v GaCi lost 11.53%, 17.54%, and 16.48% on days 1, 2, and 3 post-infection. In the control group, mice treated with HEC once-daily lost a median of 12.57%, 19.75%, and 18.45% of their infection-day body weight on the same respective days (Fig. S3).

Histopathology of wounds
Mice were sacrificed 15 days post-infection and wound beds preserved to evaluate histopathology (Fig. 5). Both BID (5A), and OD (5C) HEC-treated mice displayed large wound beds with necrotic debris evident, with little indication of re-epithelialization. By contrast mice in both the 0.1% w/v GaCi BID-treated group (5B) and the 0.3% w/v GaCi OD group (5D) displayed degrees of re-epithelialization, coupled with noticeable granulation tissue with a small amount of overlying serocellular crust. The results provide preliminary evidence of wound healing and a reduction in inflammation, which is a property of gallium that has been previously investigated by others (56, 62).

**Wound bacterial burden**

The bacterial burden of placebo-treated and GaCi-treated wounds was assessed at 24 and 72 hours post-infection. At 24 hours, mice treated with HEC placebo OD had a median log_{10} CFU burden (Fig. 5) of 7.97, while mice treated with 0.1% w/v GaCi BID, or once daily 0.3% w/v GaCi had a median CFU burden of 7.53 (P=0.0044) and 6.86 (P=0.0044), respectively. After 72 hours mice treated OD in the placebo group had a median CFU burden of 7.93, while mice treated with 0.1% w/v GaCi BID had a median CFU burden of 6.81 (P=0.0016), and mice treated with 0.3% w/v GaCi OD had a median CFU burden of 6.99 (P=0.0044).

Separately, we evaluated the bacterial burden after exposure to antibiotics that KP4640 was shown to have both susceptibility and resistance (Table S2). After exposure to colistin, which was applied topically, statistically significant and large reductions in the CFU burden was observed (Fig S3). On day 1, reduction was approximately 2.5 log_{10}. In contrast,
Primaxin™ (imipenem + cilastatin) or doxycycline-treated animals that were dosed via IP injection appeared to have no reduction of bacterial burden.

**SEM analysis of biofilm formation on occlusive dressings and wound tissue.**

SEM analysis at 2500x of the overlying occlusive dressings after seven days of infection revealed a complete covering of the substratum by *K. pneumoniae* biofilm displaying a higher architecture with cell-to-cell linkage in mice treated twice-daily with HEC placebo (Fig 7a). In contrast, the occlusive dressings of mice treated twice-daily with 0.1% w/v GaCi showed no continuous substratum *K. pneumoniae* biofilm presence and little evidence of higher biofilm architecture despite some single cells being present (Fig 7b).
DISCUSSION

From the work presented in this study, it is clear that GaCi in this formulation has significant antibacterial activity in vitro (MIC$_{50}$ 0.5 μg/mL) against a number of *K. pneumoniae* clinical isolates from wounds to include the highly resistant NDM-1 strain and other carbapenem-resistant strains in vitro (Table 1). Because of the robust in vitro activity (some < 1.0 μg/mL), the efficacy of GaCi was assessed against *K. pneumoniae* in vivo. To this end, we employed our recently developed wound model of infection (61), and essentially substituted the *A. baumannii* inoculum with a strain of *K. pneumoniae*, KP4640, that we knew was virulent in other animal models of infection (our unpublished results) and isolated from a sterile tissue site, which was likely a wound infection (60) to better mimic the *K. pneumoniae* wound infections seen in both military and civilian patient populations. We believe that this speaks to the utility of our wound infection model as this study shows it can be used with another ESKAPE pathogen (our unpublished result).

Gallium salt toxicity has been well-documented in animals and humans at high dosage (41, 56); however, at doses well-below toxic levels, gallium salts have been shown to still be efficacious against bacteria in vitro (49). Further, non-intravenous applications of gallium have resulted in reduced toxicity (49, 63), and topical use of gallium salts as an anti-infective is attractive, as it maximizes local concentration while minimizing systemic exposure. Therefore, we hypothesized that gallium salts used in a topical application for *K. pneumoniae* wound infections would also have efficacy with little or no toxicity because the compound was being applied directly to the site of infection, with limited exposure of non-infected organs. Preliminary work for this study revealed that more concentrated solutions of GaCi (from 1.0 - 3.0% w/v) resulted in a noticeable neurotoxicity in mice three
days after treatment (data not shown); however, doses in a range of 0.1 - 0.3% w/v that
displayed efficacy against *K. pneumoniae* had no visible evidence of toxic effects, such as the
neurological deficit previously observed. We have shown previously that weight loss can
be an indicator of efficacy and animal health with respect to *A. baumannii* infection and
treatments with antibiotics (61). In testing other novel antibacterial approaches, we also
found this to be a useful indicator of efficacy and overall animal health (our unpublished
data). In this work, exposure to 0.3% GaCi reduced weight loss with a statistical
significance (Fig. S2) and suggests this dosage is non-toxic. That said, we did not a
thorough analysis of the toxicity of GaCi at the 0.1% - 0.3% dosages utilized in this study.

While both GaCi concentrations tested displayed a relatively small but statistically
significant impact (0.5 to 1.5 log CFU reduction) on bacterial load (Fig. 6), we did observe
evidence of improved wound healing by measuring wound area over time (Fig. 2, Fig. 3).
Both of the wound area and bacterial burden experimental outcomes also appeared to be
dose-dependent (Fig. 2, Fig. 3, Fig 6). Further, wounds treated with GaCi displayed less
purulent discharge and inflammation at the wound edge when compared to untreated
wounds (Fig. 2, Fig. 5). While these are not quantitative measures, it is consistent with
other reports where the application of gallium salts reduced inflammation (62, 64, 65).
Further, histopathology results coincided with the gross pathology results where re-
epithelization of the wound bed was positively impacted by GaCi therapy when compared
to untreated controls (Fig. 5). Therefore, to our knowledge, this is the first study that has
shown efficacy of a gallium (III) salt against *K. pneumoniae* infection, and therefore, further
investigations of other gallium formulations and treatments against this bacterial species
are warranted especially given its multidrug resistance and prevalence in the hospital environment.

It should be noted that the while the bacterial-burden results were modest with only 0.5-1.5 log reductions in CFU of *K. pneumoniae* (Fig. 5), the impact of GaCi on the biofilm was clearly evident on the Tegaderm dressing (Fig. 6), which may contribute to the positive effect on wound healing. GaCi also exhibited some efficacy in the mouse model of *A. baumannii* wound infection with similar effects on biofilm and CFU reduction (our unpublished data), and Aridis scientists evaluated the efficacy of GaCi against *A. baumannii* infection in a porcine full thickness wound model with encouraging results (manuscript in preparation). Reports have been published using gallium nitrate against a variety of bacteria to eradicate or disperse biofilms as well as significantly reduce bacterial numbers (45, 48, 52). For example, Gallium maltolate was shown to have efficacy *in vitro* against *P. aeruginosa*, *Staphylococcus* species, and *Rhodococcus equi* (42, 66, 67). GaCi also exhibits single-digit MICs against isolates of many other gram negative bacterial species such as *P. aeruginosa*, *Escherichia coli*, *Stenotrophomonas maltophilia*, *A. baumannii*, and *Burkholderia cepacia* (Table S1). Therefore, it is not surprising that gallium (III) salts would also display efficacy against *K. pneumoniae* or have an effect on biofilm formation as these previous studies and our unpublished data support the results reported herein.

In conclusion, the results provide significant evidence of efficacy against *K. pneumoniae* *in vivo* and an improvement of wound healing which warrants the further development of gallium citrate formulations as a possible wound infection treatment. Further preclinical testing of these formulations and other gallium (III) formulations in other animal models of wound infection would be the next logical step. In addition to its
antimicrobial potency and anti-biofilm activity, the apparent anti-inflammatory and wound healing effects of a gallium topical treatment suggest clinically relevant applications for acute wound infections as well as chronic skin diseases such as diabetic foot ulcers and venous stasis ulcers could be possible with respect to *K. pneumoniae* infection. The utility of GaCi as a broad-spectrum prophylactic to prevent SSTIs rather than antibiotic prophylaxis treatment should also be considered if more compelling data can be generated.
ACKNOWLEDGEMENTS

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REFERENCES


Table 1 - MIC of gallium and antibiotics against *K. pneumoniae* strains.

<table>
<thead>
<tr>
<th><em>K. pneumoniae</em> isolate#</th>
<th>Minimal Inhibitory concentration (ug/mL)²</th>
<th>GaCl</th>
<th>Tobramycin</th>
<th>Aztreonam</th>
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<td>&gt;8</td>
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²The MIC of tobramycin or aztreonam was not determined (N.D.) for KP4640.
Figure 1 - Gallium (III) salts structures

Chemical structures of the commonly used gallium salts for antibacterial treatments.
**Figure 2**

<table>
<thead>
<tr>
<th>HEC BID</th>
<th>GaCi 0.1% BID</th>
<th>HEC OD</th>
<th>GaCi 0.3% OD</th>
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**Figure 2 - Gross Pathology**

Photographs of gross pathology of full-thickness wound over time in representative BALB/c mice. Panels A-D show full dorsal wounds on Day 7. Panels E-H show wounds on Day 15 post-inoculum. These images are representative of treated animals with HEC BID or OD (negative controls) or HEC with GaCi 0.1% BID or GaCi 0.3% OD from three separate experiments with 12 mice per group.
Figure 3 - Wound area over time (0.1% GaCi vs. HEC alone control) Box and Whisker plots show wound sizes on days 0, 6, 9, and 15 post-infection where each wound was treated with HEC alone BID (negative control) or HEC with GaCi 0.1% BID for 3 days. Boxes show median and interquartile ranges, while whiskers represent 95% CI. Treatment groups were compared each day via Mann-Whitney U-test; * represents a $p$-value of 0.05. These data are pooled from three separate experiments with 12 mice per group, 48 mice per test condition.
Figure 4

Box and Whisker plots show wound sizes on days 0, 6, 9, and 15 post-infection where each wound was treated with HEC alone once-daily (OD) or HEC + GaCi 0.3% OD for 3 days. Boxes show median and interquartile ranges, while whiskers represent 95% CI. Treatment groups were compared each day via Mann-Whitney U-test; *** and **** represent \( p \)-values of 0.001 and 0.0001 respectively. These data are pooled from three separate experiments with 12 mice per group, 48 mice per test condition.
Figure 5 - Photomicrographs of hematoxylin and eosin stained, dorsal wound longitudinal sections.

Panels A-D show wounds on Day 15 post-inoculum at 12.5x magnification view. Top panels were treated with (A) HEC BID treated or (B) GaCi0.1% BID. Bottom panels were treated with (C) HEC OD treated, (D) GaCi0.3% OD treated. Arrows delineate the perimeter of the wound. These micrographs are representative of at least 10 images taken from samples of three mice per group per test condition from three biological replicates.
Figure 6

Box and Whisker plots of log10 CFU/4mm² punch on days 1 and 3 post-inoculum. Mice were treated with HEC alone OD or HEC + GaCi at 0.3% OD for 3 days, or mice were treated with HEC alone BID or HEC + GaCi at 0.1% BID for 3 days. Boxes show median and interquartile ranges, while whiskers represent 95% CI. Groups were compared each day via Mann-Whitney U-test followed by Bonferroni correction; ** represent p-values of 0.01. These data are pooled from three biological replicates with 12 mice per group, 48 mice total per test condition.
Figure 7 – SEM analysis of occlusive dressing

Scanning electron microscopy images of Tegederm occlusive dressings 7 days post-infection. The top panel (A) shows a mouse treated with HEC BID, while the bottom panel (B) shows a mouse treated BID with 0.1% GaCi. All images are at 2500x magnification. These micrographs are representative of three biological replicates where at least 10 images were taken from each sample with three mice total per group and per test condition.