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Dual Gallium Drug Treatment Against Carbapenem-Resistant *Klebsiella Pneumoniae*: Efficacy and Potential Mechanism(s) of Action and Resistance

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Klebsiella pneumoniae **(KLP) is a Gram-negative pathogen that can be highly antibiotic-resistant. Our group has worked with gallium-based compounds as a means of treating bacterial infections. Here the possible mechanism is investigated for dual therapy comprised of gallium nitrate ($Ga(NO_3)_3$) and gallium protoporphyrin (GaPP) on KLP. It is found that in vitro the combination of $Ga(NO_3)_3$ and GaPP is synergistic against KLP. The in vivo efficacy is of the dual therapy is additionally tested by treating pulmonary KLP infections in mice. Much greater effectiveness are observed in bacterial clearance and survival of mice receiving the dual therapy than that of singly treated or untreated mice. It is found that in in vitro the dual therapy increased reactive oxygen stress in treated bacteria. Combination therapy impacted KLP catalase, but not superoxide dismutase (SOD) activity. Finally, alterations in KLP genes encoding 6-phosphogluconate phosphatase or cytochrome C assembly protein are found to be associated with increased resistance to combination gallium therapy, raising the MIC to both $Ga(NO_3)_3$ and GaPP by 4-fold. These cumulative data lend validation to the potential for the use of $Ga(NO_3)_3$ and GaPP combination therapy against KLP and suggest that increased oxidative stress is involved in the mechanism of action.**

1. Introduction

Klebsiella pneumoniae (KLP) is a Gram-negative bacterium found as part of the naturally occurring microflora in/on our mouths, skin, and lower intestines where it does not cause disease.[1] However, if it gains access to other locations, such as the lungs, open wounds, or the urinary tract, the bacterium acts as a pathogen, potentially causing local wound infections, pneumonia, urinary tract infections, or septicemia.[1] In recent years, strains of KLP with resistance to increasing numbers of antibiotics have emerged. Carbapenem-resistant KLP is of particular concern as carbapenems are a class of antibiotics typically used in the case of a severe bacterial infection resistant to other antibiotics, often as a last line of therapeutic defense.[2] Resistance to carbapenems could leave patients with very few, or potentially even no, options available for the treatment of such infections.

With KLP being one of the more common causes of nosocomial infections, finding new treatments of KLP is a high priority for the medical community.[3] However, even when new antibiotics are discovered, pathogens can develop resistance rather quickly, sometimes in as little as one year.[4] One approach to combat the emergence of drug-resistant strains of bacteria is to manipulate or inhibit essential functions of bacteria, as alterations of these systems cannot be easily made without affecting the organism's fitness.

Iron is an essential element for most living beings, as it plays a critical role in a wide variety of biological functions, including cellular respiration, DNA transcription/translation, and defense against oxidative stress.[5] The ability of iron to redox cycle between divalent and trivalent oxidation states is required to perform these functions. Trivalent gallium (Ga^{3+}) is similar to trivalent iron and has a similar ionic radius, allowing gallium to be substituted for iron in the active site of some of these enzymes. However, under physiological conditions, gallium cannot undergo reduction to the divalent state.[6] Thus, should a bacterium take up gallium and incorporate it into a metalloprotein in place of iron, that protein would no longer function, potentially leading to growth inhibition or the death of the bacterium.[7]

We have previously performed pilot studies using a combination of different gallium compounds to treat KLP which showed synergistic antimicrobial activity. The combination of $Ga(NO_3)_3$ and gallium protoporphyrin (GaPP) significantly disrupted KLP biofilms and increased the survival of *C. elegans* infected with KLP. As such, we wished to further investigate the capabilities

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Table 1. Minimum inhibitory concentration (MIC) and Fractional inhibitory concentration (FICIs) against *K. pneumoniae* ATCC BAA 1705.

Strain	MIC [μg mL^{-1}]		FICI ($Ga(NO_3)_3$/GaPP, μg mL^{-1})
	$Ga(NO_3)_3$	GaPP	
K. pneumoniae	4[a]	16[a]	0.26 (1/0.125)[a]
K. pneumoniae ΔkatE	512[b]	1[b]	N/A
K. pneumoniae[c]	16[a]	64[a]	N/A

[a] BM2 Medium, [b] BM2 Medium containing 10% Tryptic Soy Broth (TSB), [c] Ga drug-resistant strain. FICI ≤ 0.5: Synergism, FICI ≥ 3: Antagonism, 0.5 < FICI < 3: Additive.

of gallium compounds against carbapenem-resistant KLP and to further delineate the mechanism(s) of action of combination gallium therapy.[8] Work from our group and others has increased oxidative stress as a downstream effect of gallium compounds in microorganisms.[9] As such we explored the role of reactive oxygen species (ROS) and oxidative stress in the efficacy of dual gallium therapy against KLP.

2. Results

2.1. In Vitro Synergism of Combination Gallium Therapy

We performed microbroth dilution assays and checkerboard assays to determine/confirm the MICs of $Ga(NO_3)_3$ and GaPP and the Fractional Inhibitory concentration Indexes (FICIs) against carbapenem resistant KLP ATCC BAA 1705 strain. The FICI is the sum of the ratios of the MICs of the compounds individually divided by the MICs when the compounds are used together and is used to assess their synergism (**Table 1**).[8a,10] We observed an MIC of 4 and 16 μg/mL for $Ga(NO_3)_3$ and GaPP, respectively, and an FIC of 0.26 using 1 μg ml^{-1} $Ga(NO_3)_3$ and 0.125 μg mL^{-1} of GaPP. These results align with our previously reported values showing that gallium compounds and especially the combination $Ga(NO_3)_3$ and GaPP are effective against carbapenem-resistant KLP.[8a]

2.2. Efficacy of a Gallium Combination in a Murine KLP Lung Infection Model

Given the synergism of combination $Ga(NO_3)_3$ and GaPP therapy seen in vitro, we tested its efficacy in vivo in a murine pulmonary infection model. Four groups of mice, consisting of 3 mice in the singly treated sets, and 6 mice in the untreated and combination-treated groups, were intranasally infected with KLP (8×10^8 CFU per mouse). One-hour post-infection, the mice were treated intranasally (IN) with GaPP, $Ga(NO_3)_3$, a combination of GaPP and $Ga(NO_3)_3$, at 10 mg per kg of body weight, or a mock treatment with PBS, every 24 h for 2 days. To increase the ability to detect an enhanced effect of combination therapy, we used suboptimal concentrations of the individual compounds, as previously determined.[11] Over the course of 48 hours, deaths were seen in the untreated and singly treated mice (**Figure 1B**). Conversely, the combination-treated mice had a 100% survival rate (Figure 1B).

At 72 hours, the surviving mice were sacrificed, and lungs extracted to determine both bacterial burden and histological changes. One lung lobe was mechanically homogenized in PBS and serially diluted to determine the CFU present in the lungs. As observed in Figure 1A, the bacterial burden in the combination-treated mice was significantly reduced compared to that of untreated and $Ga(NO_3)_3$-treated mice, but not that of the GaPP-treated mice.

The second lobe of each surviving mouse was fixed and stained with Gram and H&E stain to assess the extent of infection and tissue damage, respectively. As shown in **Figure 2**, the singly treated and untreated samples demonstrated numerous bacteria (circled), whereas the combination-treated samples showed a lesser number of bacteria in the lungs. This correlates with the CFU counts (Figure 1B). We also observed severe and diffuse acute pneumonia distribution in singly treated and untreated mice lung read by blinded pathologist. However, combination-treated lung tissue showed patchy and moderate acute pneumonia distribution that correlates with the survival study where all mice are alive in combination-treated (Figure 1A). A longer treatment period is potentially necessary to eradicate all the bacteria

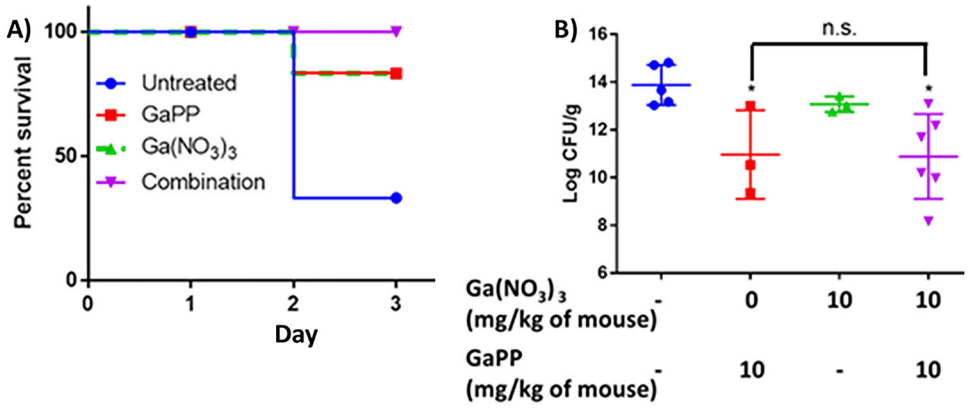

Figure 1. Synergy of gallium compounds in murine KLP lung infection. A) Percent survival. B) Bacterial load in the lung. Mice were infected IN (8×10^8 CFU/mouse) and treated (10 mg/ kg) via IN administration for 2 days. Negative control mice received sterile PBS instead of a gallium compound via IN administration. Treated mice received 10 mg/ kg of each gallium compound both individually and in combination. Significant differences were determined using One-way Anova (n = 3 for single drug treatment, n = 6 for untreated control and combination-treated). Data represent mean ± standard deviation. *p = 0.0137, n.s.: no significance.

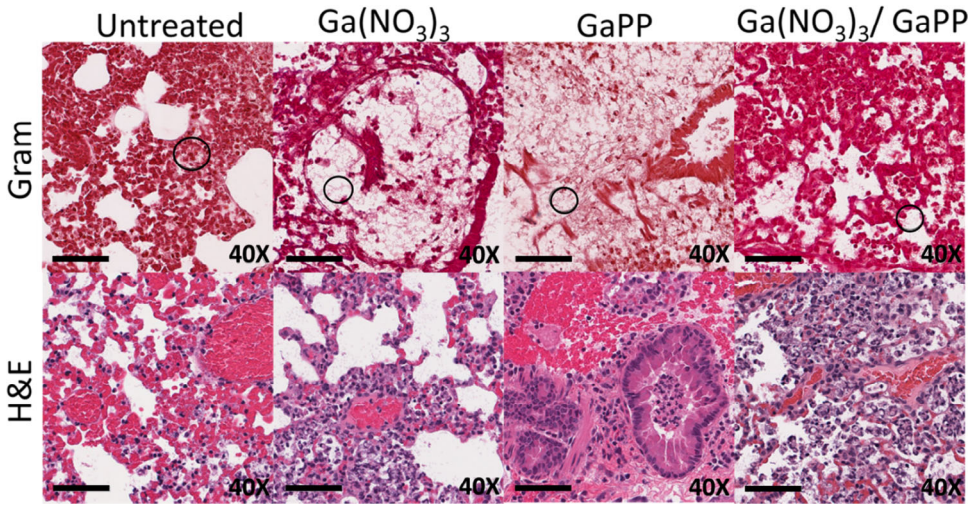

Figure 2. Effect of gallium treatment on lung Gram stain and histology of murine pulmonary KLP infection. Mice were infected IN with KLP and then received gallium IN 1-hour post-infection in the form of GaPP, $Ga(NO_3)_3$, or a combination of GaPP and $Ga(NO_3)_3$, at 10 mg /kg of body weight every 24 h for 2 days. Untreated mice received PBS. All mice were sacrificed after 2 days of infection and treatment, following which sections of lung underwent tissue Gram stain (top row) and H&E staining (bottom row), with representative results shown for each treatment. The gray bars represent 60 µm.

from mice lung and further resolve the acute inflammation observed. Nevertheless, based on these results (Figures 1 and 2), combination gallium therapy is efficacious in murine lung KLP infection.

2.3. Gallium Induces Reactive Oxygen Species (ROS) Accumulation

Previous work provided strong support for the bacteriostatic and bactericidal effects of gallium compounds being due at least in part to the inhibition of bacterial antioxidant enzymes and/or electron transport disruption, leading to an accumulation of ROS.[12] Therefore, if this process were occurring in gallium-treated KLP, we would expect to see an accumulation of ROS.

To test for ROS, we treated KLP with increasing concentrations of GaPP or $Ga(NO_3)_3$ alone and in combination after loading KLP with H2DCFDA. H2DCFDA is converted to a fluorescent molecule in the presence of ROS. We then monitored and compared the various samples' fluorescence over 24 hours (**Figure 3**). We observed that at MIC and sub-MIC concentrations, the drug-treated bacteria produced a greater amount of fluorescence than their untreated counterparts, reflecting an increase in ROS. However, presumably related to bacterial death, ROS levels decreased when drug concentrations exceeded the MICs. In the combination-treated samples, all concentrations showed an accumulation of ROS that was similar to or greater than, the singly treated samples despite the much lower drug concentrations employed.

2.4. Nature of ROS in Gallium-Treated Bacteria

As shown in Figure 3, we observed that in both singly and combination-treated bacteria there was an accumulation of ROS. We next sought additional insight as to the specific ROS

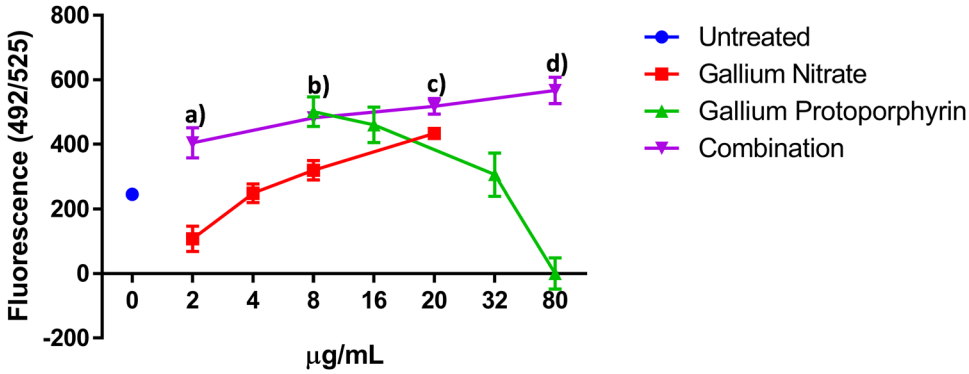

Figure 3. Accumulation of ROS within KLP by GaPP and $Ga(NO_3)_3$. ROS within KLP was measured using H2DCFDA. The bacteria were incubated with GaPP or $Ga(NO_3)_3$ for 5 h, following which they were harvested and incubated with H2DCFDA (10 µM) for 30 min after which fluorescence measurements were obtained. Shown are fluorescence measurements with (0, blue dot): KLP with H2DCFDA or increasing concentrations of $Ga(NO_3)_3$ (red line) or GaPP (green line). Also shown in purple are fluorescent values when KLP were exposed to the combination of a) 0.0625 µg ml^{-1} GaPP and 0.5 µg ml^{-1} $Ga(NO_3)_3$, b) 0.125 µg ml^{-1} GaPP and 1.0 µg ml^{-1} $Ga(NO_3)_3$, c) 0.25 µg ml^{-1} GaPP and 2 µg ml^{-1} $Ga(NO_3)_3$, and d) 0.625 µg ml^{-1} GaPP and 5 µg ml^{-1} $Ga(NO_3)_3$. Data are the mean ± standard deviation (n=4).

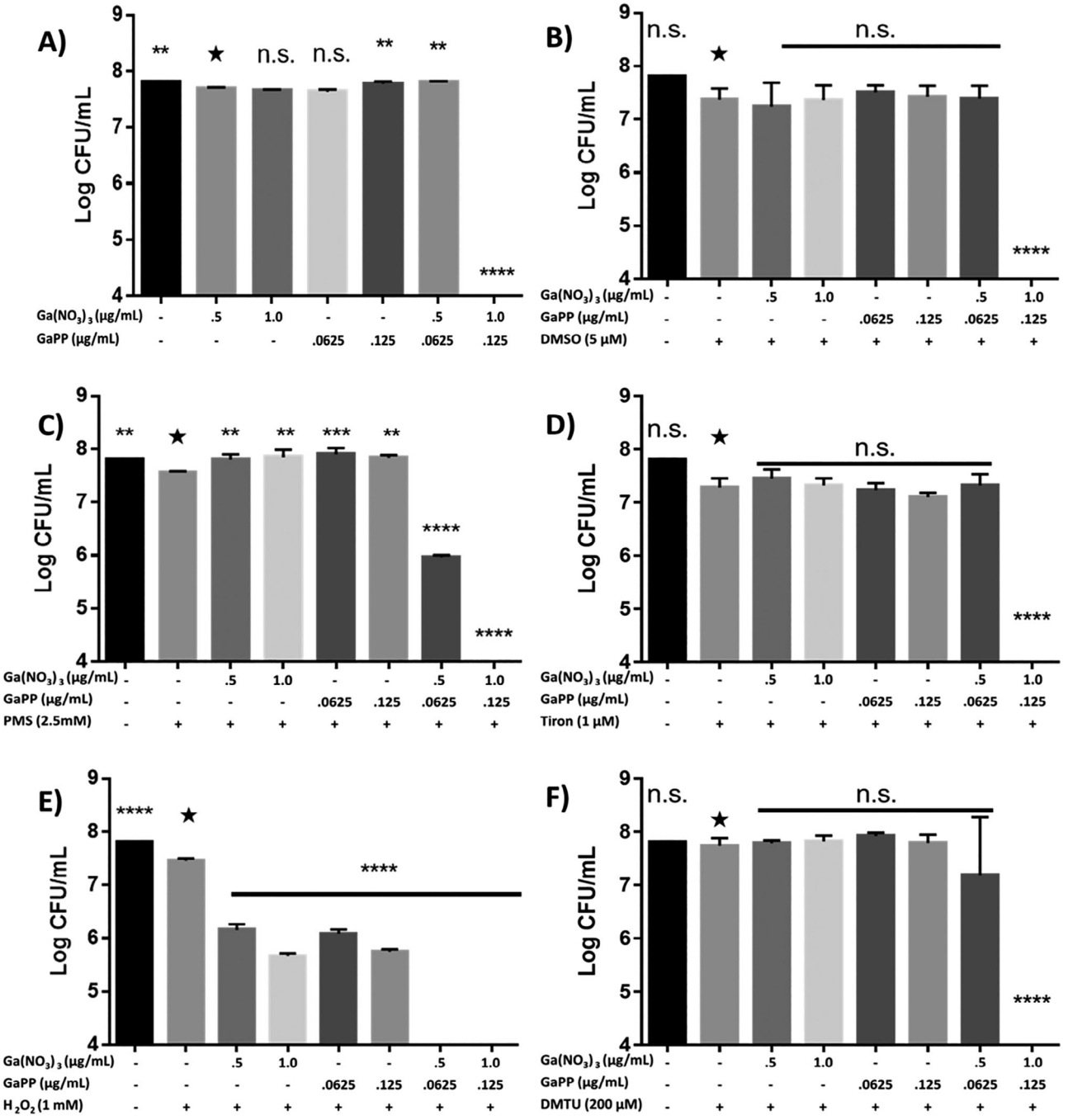

Figure 4. Effect of pro-and antioxidant compounds on the growth of gallium-treated KLP. An overnight culture of KLP was subcultured at a concentration of 4 x10^6 CFU mL^{-1} in the absence or presence of the gallium compounds for 20 hours, shaking at 37°C. After washing with PBS twice, KLP was subcultured again at a concentration of 1x10^7 CFU mL^{-1} for 1 hour in BM2 media in the absence (panel A) or presence (panels B-F) of the pro-oxidant or antioxidant compounds indicated: B) treated with 5 μM DMSO, C) treated with 2.5 mM PMS, D) treated with 1 μM Tiron, E) treated with 1 mM H_2O_2, F) treated with 200 μM DMTU. Data represent mean ± standard deviation (n = 3). Significance was determined via One-way ANOVA (Prism 6.0, Graphpad) compared to untreated, ****P < 0.0001, ***P < 0.001 **P < 0.01, n.s.: no significance. ★ indicates the untreated control column that other columns were compared to for one way analysis.

generated and their role in antimicrobial activity by assessing the impact of a variety of well-characterized prooxidants and antioxidants on gallium-mediated antimicrobial activity.

Gallium treatment of KLP was undertaken along with exposure to one of two prooxidants, phenazine methosulfate (PMS), an inducer of superoxide production (**Figure** 4C), or exogenous hydrogen peroxide (H_2O_2) (Figure 4E), or one of three antioxidants, DMSO, a hydroxyl radical scavenger, disodium 4,5-dihydroxy-1,3-benzenedisulfonate (Tiron), a superoxide radical scavenger, or dimethyl thiourea (DMTU), a H_2O_2 scavenger,

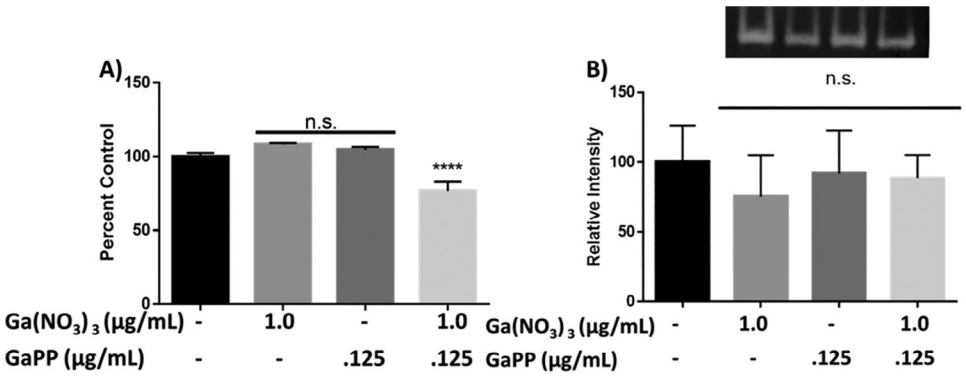

Figure 5. Effect of gallium combination therapy on KLP SOD activity. KLP was incubated overnight with $Ga(NO_3)_3$, GaPP, or both. Bacterial lysate was then collected from washed bacteria and assayed for SOD activity via A) activity kit or B) native gel activity assay. The insert in panel B shows the bands which were found to be consistent with MnSOD. Densitometry of these bands was then measured for each treatment condition. Significance was determined via One-way ANOVA compared to untreated. ****$P < 0.0001$. A small but modest decrease in SOD activity was seen with combination gallium therapy using the activity kit but could not be confirmed by activity gel assay, where no difference in MnSOD band intensity was seen.

(Figure 4B,D, and F). Concentrations of PMS and H_2O_2 that were sublethal for the bacteria were chosen to be able to assess the interaction between gallium exposure and the prooxidants. CFUs were determined at 20 hours and compared to a pro/antioxidant free control (Figure 4A).

We observed that gallium treatment, both single agent and combination, significantly increased the sensitivity of KLP to H_2O_2 (Figure 4E). Combination, but not single agent, therapy increased KLP sensitivity to superoxide (PMS treatment, Figure 4C), recognizing that PMS will also result in the generation of H_2O_2 via the spontaneous or SOD-enhanced dismutation of superoxide. This enhanced sensitivity to ROS with gallium treatment could enhance the susceptibility of KLP to phagocyte-mediated killing, as part of that process involves phagocyte generation of superoxide and H_2O_2.

We also observed that each of the three antioxidants was able to recover the growth of the treated KLP, and H_2O_2 significantly reduced the growth of all gallium-treated KLP samples (Figure 4B,D, and F). These data are consistent with the important role of elevated levels of H_2O_2 contributing to the antimicrobial activity of gallium combination therapy.

2.5. SOD Activity of Gallium-Treated KLP

Superoxide dismutases (SOD) are a group of metal co-factored antioxidant enzymes that catalyze the degradation of superoxide to oxygen and H_2O_2.[13] SOD functions by shuttling electrons between a metal cofactor and the radical making it an excellent target for gallium therapy. KLP can express three different SOD isoforms, each with its own metal cofactor, Mn, Fe, or Cu/Zn, encoded by sodA, sodB, and sodC, respectively.[14] This differs from most bacteria, which express only two, MnSOD and FeSOD.

Given the increased susceptibility of KLP to PMS seen above (Figure 4E), we examined the impact of single and combination gallium therapy on KLP SOD activity using two different assays. As shown in **Figure** 5A, as measured by a commercial SOD activity kit, we found no significant difference in total SOD activity among untreated KLP and those treated with singly with $Ga(NO_3)_3$ or GaPP. A modest but significant decrease in SOD activity was seen with a combination of the two gallium compounds.

To assess which of the various SOD isoforms that KLP is capable of expressing was impacted by gallium, SOD activity of KLP lysates were assessed by SOD activity gel. Surprisingly in 10% TSB media, only a MnSOD band was seen, and we were not able to detect a significant difference in SOD activity among the untreated control and either singly or combination-treated KLP (Figure 5B).

The inability to detect a decrease in SOD activity with combination gallium treatment seen previously in the biochemical activity assay may be due to the less quantitative nature of the activity gel methodology. Nevertheless, these data do not point to alterations in SOD activity as playing a major role in KLP susceptibility to gallium or the increase in steady-state ROS noted above (Figure 3).

2.6. Catalase Activity of Gallium-Treated KLP

As seen in Figure 4E, gallium therapy resulted in a greater sensitivity of KLP to H_2O_2 than PMS-induced superoxide, implying that these compounds target impact clearance of H_2O_2. Inhibition of catalase, an iron-containing enzyme responsible for the breakdown of H_2O_2 into water and oxygen, therefore seemed a potential target for gallium therapy. Accordingly. we investigated the effect of these gallium compounds on catalase activity via two different methods, native gel activity assay (**Figure** 6A) and spectrophotometry activity assay (Figure 6B,C).

As shown in Figure 6A, the native gel assay found that $Ga(NO_3)_3$ and the combination of $Ga(NO_3)_3$ and GaPP led to a significant decrease in KLP catalase activity, but GaPP had no significant effect. These results were also observed using a spectrophotometric biochemical assay (Figure 6B). In both assays, we observed reduction in catalase activity with combination therapy that seemed to be primarily due to the action of $Ga(NO_3)_3$, as the impact of the combination of $Ga(NO_3)_3$ and GaPP was not significantly different than that of $Ga(NO_3)_3$ alone. To assess if the lack of effect of GaPP was concentration related (Figure 6A,B), experiments were repeated using higher concentrations of GaPP.

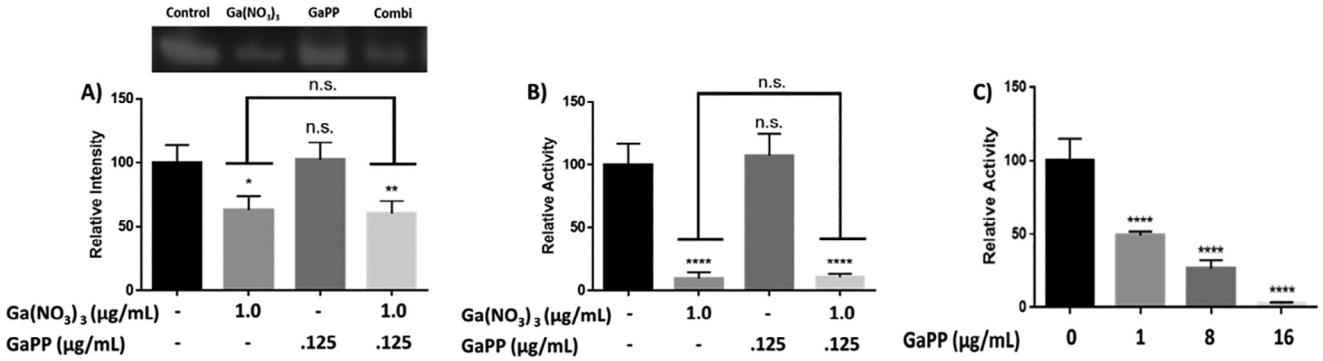

Figure 6. Effect of gallium combination therapy on KLP catalase activity. KLP was incubated overnight with $Ga(NO_3)_3$, GaPP, or both. The bacterial lysate was then collected from washed bacteria and used to determine A) relative catalase activity from a native gel. B) catalase activity from spectrophotometry assay, C) catalase activity after treatment with increasing concentrations of GaPP using spectrophotometry assay. One-way ANOVA was used to determine a significant difference compared to untreated. ns: non significance, ****$P < 0.0001$, ***$P < 0.001$, **$P = 0.0035$. In both assays, we observed a reduction in catalase activity with combination therapy that seemed to be primarily due to the action of $Ga(NO_3)_3$,.

These data (Figure 6C) revealed that when higher concentrations, even sub-MIC concentrations, were used significant reduction in catalase activity did occur.

2.7. Creation of Catalase Knockout Mutants

Given the effect of gallium treatment on catalase activity, we assessed whether the absence of catalase would alter the susceptibility to KLP to GaPP or $Ga(NO_3)_3$. Catalase knockout mutants were constructed from the KLP WT strain as previously described by Huang et al.[15] The process began with the generation of a knockout cassette containing an apramycin resistance gene and sequences up and downstream of our desired knockout targets, *katE*. This cassette was then inserted in competent KLP via electroporation, where the homologous sequences allowed for allelic exchange to replace the targeted genes with the knockout cassette, conferring resistance to apramycin as a selection marker. The mutants were then checked via PCR and agarose gel.

MIC determination was then employed to investigate the resistance of the mutants towards GaPP and $Ga(NO_3)_3$. The knockouts exhibited no growth in BM2 medium (Table 1). However, KLP Δ*katE* grew in BM2 medium containing 10% tryptic soy broth (3 g L^{-1}). The MIC of the $Ga(NO_3)_3$ against Δ*katE* strain increased markedly relative to the parent strain, 512 μg mL^{-1} versus 4 μg mL^{-1}, respectively (Table 1) In contrast the Δ*katE* strain was much more susceptible than the parent to GaPP, with an MIC of 1 μg mL^{-1} compared to 16 μg mL^{-1} for the parent strain (Table 1). These data suggest that catalase is important in KLP susceptibility to both gallium compounds.

2.8. Spontaneous Mutation of KLP

To identify other possible genes that could contribute to the resistance of KLP to GaPP/$Ga(NO_3)_3$, we used a spontaneous mutation technique. Initially, KLP was cultured in the presence of various concentrations of GaPP/$Ga(NO_3)_3$. After sixteen sequential sub-cultures of growing KLP in a medium containing the highest concentration of GaPP/GN, the culture with inhibiting growth was plated on TSA plates, and colonies were selected to assess the resistance degrees. A MIC was determined in the BM2 medium. The mutant was resistant to GaPP and $Ga(NO_3)_3$, exhibiting MICs of >64 μg ml^{-1} and 16 μg ml^{-1}, respectively (Table 1). These MICs are 4 times higher than the MICs against the wild-type strain.

The genomic DNA from the resistant mutant was extracted and sequenced using Next-generation sequencing (NGS) to find genes responsible for the resistance to GaPP and $Ga(NO_3)_3$. Two genes were found with allele changes in a single position in a protein-coding region compared to a reference; genes encoding 6-phosphogluconate phosphatase (NCBI locus_tag: KPBAA1705_13 790) or cytochrome C assembly protein (NCBI locus_tag: KPBAA1705_21 476) (**Figure 7**).

3. Discussion

Studies from our group and others have shown that gallium compounds are effective in treating bacterial, viral, and even fungal infections.[8b] These compounds typically work by integrating gallium into metalloproteins that contain an iron or heme cofactor. This causes the proteins to become non-functional gallium cannot undergo redox chemistry required for their function as under physiological conditions.[8a,16] Our group has previously investigated the effects of a gallium dual therapy composed of $Ga(NO_3)_3$ and GaPP on both *Pseudomonas aeruginosa* and KLP. In this study, we expanded our understanding of the use of this therapy against carbapenem-resistant KLP both in vitro and in vivo.[8a,c]

We performed microbroth dilution and checkerboard assays to determine the effectiveness of the gallium compounds alone and in combination. Consistent with our prior work, $Ga(NO_3)_3$ and GaPP exhibited MICs against KLP of 4 μg ml^{-1} and 16 μg mL^{-1}, respectively. These MICs are similar to the 1 μg mL^{-1} and 16 μg mL^{-1}, respectively, seen in *P. aeruginosa*.[8c]

Furthermore, the combination therapy of $Ga(NO_3)_3$ and GaPP had an FIC of 0.26, suggesting that it is synergistic against KLP.[8c] The observed synergism may stem from the combination therapy's ability to target both the elemental iron and heme metabolism through $Ga(NO_3)_3$ and GaPP, respectively, allowing

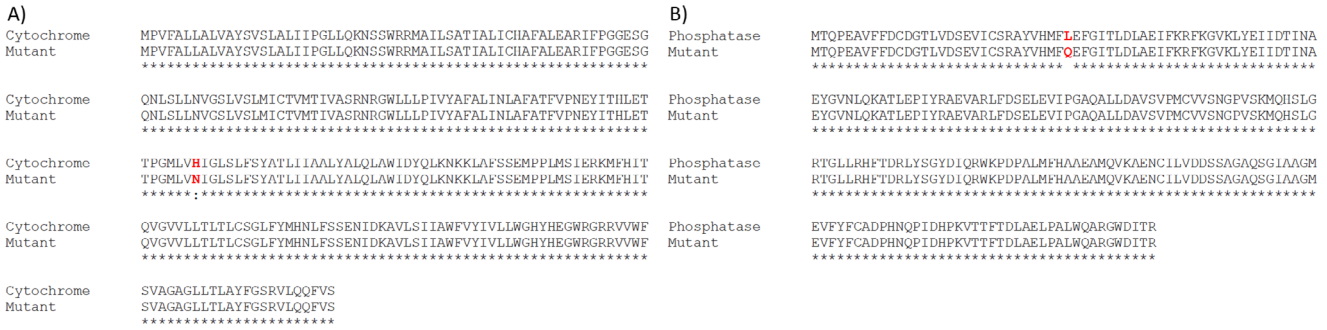

Figure 7. Protein sequence alignment. A) cytochrome C assembly protein (NCBI locus_tag: KPBAA1705_21476), B) 6-phosphogluconate phosphatase (NCBI locus_tag: KPBAA1705_13790).

for an impact on a wider variety of targets than either compound alone.

We also tested combination gallium therapy in vivo in a murine lung infection model. We found that singly treated GaPP mice and combination-treated mice had reduced but statistically similar levels of CFU in their lungs compared to that of the untreated and singly $Ga(NO_3)_3$ treated mice. However, only the combination treatment prevented death of infected mice. This indicates that the combination treatment provided a protective effect against death that is separate from its ability to reduce CFU concentration in the lungs. This is seemingly corroborated by lung histology. Gram and H&E-stained lung sections, regardless of treatment or lack thereof, demonstrated widespread infection and significant pneumonia tissue damage, suggesting that if the combination is to work, a longer treatment period (e.g., 7 days) for the combination therapy may be required for KLP lung infection resolution.

Previous research by our group and others, suggests that oxidative stress plays a key role in the killing action of gallium compounds.[8b,c] Consistent with this prior work, using the redox-sensitive probe H2DCFDA, we found that both $Ga(NO_3)_3$ and GaPP-treated KLP accumulated more ROS than the untreated bacteria. Not surprisingly, using concentrations much smaller than what was used in the singly treated samples. KLP treated with the combination of both gallium drugs accumulated more ROS than either single treatment. When bacteria take up gallium and they may incorporate it into metalloproteins in place of iron, which results in a non-functional protein. Such proteins can be in the electron transport pathway, blocking electron transport and leading to univalent reduction of molecular oxygen to form superoxide and ultimately hydrogen peroxide. Thus, increased production of ROS. In addition, several antioxidant enzymes, such as catalase and iron superoxide dismutase, can also potentially be negatively impacted by iron/heme substitution with gallium, resulting in a decrease in ability to catabolize ROS. The overall result through both increased production and decreased catabolism would be an increase in steady state levels of ROS.[7]

After determining that significant levels of ROS were being accumulated in the drug-treated bacteria, we set out to determine the nature of ROS produced and which proteins, if any, are being affected by gallium treatment. To do this, we subjected gallium-treated bacteria to exogenous oxidants and antioxidants. In these experiments DMTU, Tiron, and DMSO, which are H_2O_2, superoxide, and hydroxyl radical scavengers respectively, were all able to successfully recover the growth of the gallium-treated bacteria to levels comparable to non-gallium-treated bacteria (Figure 4). As previously found with *P. aeruginosa*, we observed that each antioxidant was able to return the growth of gallium-treated KLP to that of the non-gallium-treated control.[8c] However, different from prior results with *P. aeruginosa*, PMS did not reduce KLP growth in singly treated samples, while H_2O_2 significantly reduced the growth of all gallium-treated KLP samples. This also implied that the gallium treatment targets catalase in KLP, and potentially to a much lesser extent, the combination may be targeting SOD in KLP.

We tested the overall SOD activity of gallium-treated KLP using a SOD activity assay kit. Combination therapy had a modest but significant reduction in total SOD activity (Figure 5A). However, a native gel activity assay for SOD isoenzyme determination failed to show any difference, with almost all activity due to Mn-SOD, the combination therapy did not show any significant difference. Guo et al previously investigated the effect of $Ga(NO_3)_3$ treatment on SOD and catalase protein expression in the *K. pneumoniae* ATCC 43816 strain and observed transcriptional levels of two SOD-encoding genes and two catalase-encoding genes were downregulated.[9b] However, their enzymatic activity were not measured.

Hydrogen peroxide significantly reduced the growth of both singly and combination gallium-treated KLP (Figure 4). This implies that gallium may be targeting and inhibiting the action of catalase, which would be in agreement with a prior work by Guo et al.[9b] In our experiments, we saw that both the combination-treated and the singly $Ga(NO_3)_3$ treated bacteria had a significant reduction in catalase activity in both the native activity gel and spectrophotometric biochemical assay, while GaPP alone had no significant effect on catalase activity at low concentration. Combination treatment didn't fare any better than $Ga(NO_3)_3$ alone in catalase inhibition. Further experimentation revealed that the lack of reduction observed in GaPP treatment was a dose-dependent effect. As seen in Figure 6C, a combination of 5 μg mL^{-1} of $Ga(NO_3)_3$ and 20 μg mL^{-1} of GaPP did reduce activity. These results for the most part correlate with what was observed in Figure 4 and in the research by Guo et al. that $Ga(NO_3)_3$ led to a downregulation of both KLP catalase genes.

These KLP catalase data run contrary to our prior observations in *P. aeruginosa*. $Ga(NO_3)_3$ was unable to reduce catalase activity at concentrations as high as 30 μg mL^{-1}. GaPP on the other hand, did decrease catalase activity, but much higher GaPP

concentrations were needed to achieve the level of activity reduction in *P. aeruginosa* seen at just 1 µg mL^{-1} in KLP.[8c]

We additionally examined engineered KLP strains derived from ATCC BAA 1705 that had knocked out one of the catalase genes in the strain, KatE. Knockout of *KatE* gene drastically decreased the growth in BM2 medium. Mutations, including knockouts, can have wide-reaching effects beyond what was intended by the alterations. However, it is known that KLP only possesses two catalase genes, *katE* and *katG*. In theory, by removing one, we should make it more susceptible to H_2O_2 stress, and inhibition of the remaining catalase should further increase that susceptibility. Therefore, the GaPP should be more effective at lower concentrations. However, this would not account for the increase in resistance to $Ga(NO_3)_3$, which also decreased catalase activity in our experiments and those performed by others. Further work is required to elucidate the mechanism(s) responsible for these MIC changes.[17]

In addition to a role for catalase in KLP resistance to combination gallium therapy, a spontaneous mutation protocol led to the identification of two additional genes, 6-phosphogluconate phosphatase (NCBI locus_tag: KPBAA1705_13 790) or cytochrome C assembly protein (NCBI locus_tag: KPBAA1705_21 476). It is not immediately apparent as to how these genes would be involved in gallium resistance. Additional work is required to identify the responsible mechanism(s)

4. Conclusion

Our research indicates that the dual therapy of $Ga(NO_3)_3$ and GaPP is an effective means of treating KLP infections in vitro and in vivo. We found that the combination of $Ga(NO_3)_3$) and GaPP was synergistic against KLP. The efficacy of the dual therapy by treating pulmonary KLP infections in mice was undertaken and observed much greater effectiveness in bacterial clearance and survival of mice receiving the dual therapy than that of singly treated or untreated mice. From there, we observed that the combination therapy negatively impacted catalase but not SOD activity. Finally, alterations in KLP genes encoding 6-phosphogluconate phosphatase or cytochrome C assembly protein were found to be associated with increased resistance to combination gallium therapy. The experiments performed imply that the dual therapy works by increasing the bacteria's susceptibility to oxidative stress, particularly H_2O_2-mediated stress, by inhibiting catalase activity.

5. Experimental Section

Materials and Reagents: Gallium(III) protoporphyrin IX chloride (GaPP, (> 95% purity)) was purchased from Frontier Scientific (Logan, UT, USA). $Ga(NO_3)_3$ (> 95% purity) was purchased from Acros Organics (Carlsbad, CA, USA). Dulbecco's Modified Eagle Medium (DMEM, high glucose) was purchased from GE Healthcare Life Sciences (Logan, UT, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) for GaPP or sterilized water for $Ga(NO_3)_3$ and stored at −20 °C until needed.

Strains: Carbapenem-resistant KLP (ATCC BAA 1705) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). KLP was cultured in an iron-limited BM2 medium (pH 7.0) as previously described.[9b,16] Δ*KatE* was created from this strain of KLP for these experiments. Prior to growth in iron-limited BM2 media, bacteria were maintained on Lennox Broth (LB) agar.

Creation of KLP ΔKatE Mutants: A KLP *KatE* knockout strain was prepared as previously described by Huang et al., with modification based on the work of Jeong et al.[15,18] This process began by rendering the wild-type KLP competent. This started by inoculating 100 mL of LB with 1 mL of overnight culture in a 250 mL Erlenmeyer flask at 37° C, shaking until reaching an OD_{600} of 0.2. The bacteria were then centrifuged, and the supernatant was discarded. The pellet was washed three times with 50 mL of ice-cold 10% glycerol. After discarding the supernatant from the third wash, the pellet was resuspended in the residual glycerol. The competent cells were then aliquoted into 100 uL portions in microcentrifuge tubes, flash-frozen in liquid nitrogen, and stored at −80 °C. The competent cells were then electroporated with the plasmid pACBSR-hyg with the following settings 2.5K V, 200 Ω, and 25 µF. The resulting bacteria were then rendered competent as previously described. The new competent bacteria were then electroporated with DNA from p-MDIAI containing an apramycin selection marker amplified with DNA from up and down-stream of *katE* to allow for homologous recombination to knock out those genes. The bacteria were then passed on LBA apramycin plates for 3 days and checked with PCR amplification and gel electrophoresis.

Spontaneous Gallium-Resistant KLP: An overnight KLP culture was diluted to 1:100 in BM2 media containing various concentrations of GaPP (0.5 × MIC – 4 × MIC) or GaPP/$Ga(NO_3)_3$ combination (0.5 × FIC – 4 × FIC). The cultures were incubated at 37 °C with shaking at 250 rpm until the growth was observed in the tube with the highest concentration of GaPP or the combination. The KLP culture containing the highest GaPP, or the combination, concentration was diluted to 1:100 with fresh media containing two-fold serial dilutions of GaPP or the combination. This procedure was repeated by diluting the parental culture (the highest GaPP concentration) with fresh media containing GaPP or the combination for 16 passages. Finally, a single colony was isolated from TSA plates, cultured overnight, and stored in TSB containing 10% DMSO at −80 °C.

DNA Extraction and Sequencing: The GaPP-resistant KLP was cultured overnight in BM2. DNA was extracted using the QIAamp DNA Kit (Qiagen, Germany) according to the manufacturer's manual. Sequencing and bioinformatics were done at the Core facility at the University of Nebraska Medical Center.

Antimicrobial Susceptibility Test: Minimum inhibitory concentration (MIC) was measured using microbroth dilution assays in the BM2 medium.[19] The inoculum was 0.5 × 10^6 CFU mL^{-1}. The MIC was defined as the lowest concentration of agent that inhibited visible growth at 37 °C and confirmed by measuring OD_{625nm}.

Detection of Reactive Oxygen Species (ROS) Accumulated in KLP: KLP was cultured in iron-limited BM2 medium at 37 °C and diluted to OD_{600} = 0.5 in PBS. KLP was suspended in BM2 containing 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen) dissolved in DMSO and incubated at room temperature for 1 h. After washing KLP with PBS by centrifugation at 8000 x g for 10 min, the bacteria were resuspended in 0.1 mL BM2 containing GaPP, $Ga(NO_3)_3$, both, or media alone and incubated at 37 °C for 30 minutes. The samples were then transferred to a 96-well plate, and fluorescence was measured 1-, 2-, and 24-hours post-drug administration at Ex/Em = 492/525 nm to determine ROS using a Biotek Synergy H1 Hybrid Reader (Winooski, VT, USA).

Sensitivity of KLP to Oxidants and Antioxidants Because of Gallium Treatment: The assay was performed as described in the previous work with modification.[12a] KLP (4 ×10^6 CFU/mL) were cultured overnight with or without gallium compound(s) in iron-limited BM2 media. The following day, cultures were washed twice with PBS and resuspended to concentration 1 ×10^7 CFU mL^{-1} and treated with H_2O_2 (0.5 mM), the superoxide-generating compound phenazine methosulfate (PMS, Sigma–Aldrich, St Louis, USA), the hydroxyl radical scavenger DMSO (Sigma-Aldrich, St Louis, USA), the H_2O_2 scavenger dimethyl thiourea (DMTU, Sigma–Aldrich, St Louis, USA), or the superoxide scavenger Tiron from Acros Organics or ascorbic acid (Sigma–Aldrich, St Louis, USA) at 37 °C for one hour. The cultures were then serially diluted and plated on tryptic soy agar plates to determine CFU.

SOD Activity Assay: SOD activity was determined by the colorimetric method using the SOD Assay Kit – WST from Dojindo Molecular Technologies, Inc (Rockville, MD, USA). An overnight culture of KLP was diluted to

OD_{625} = 0.1 in iron-limited BM2 medium with and without GaPP and/or $Ga(NO_3)_3$. The bacteria were then incubated overnight at 37 °C with shaking. The culture was washed twice with PBS (8000 × g for 10 min at 4 °C); the resulting pellet was suspended in a solution of PBS containing 1% B-Per (ThermoFisher Scientific, Waltham, MA, USA) and incubated at 37° C for 1 hour. Then, it washed as previously described. The resulting pellet was suspended in 50 mM phosphate buffer (pH 7.0) containing a protease inhibitor cocktail and mechanically disrupted via glass bead beating. The lysate was collected by centrifuging at 12000 × g for 20 min at 4 °C. The lysate was transferred to Spin-X centrifuge tubes and centrifuged at 5000 x g for 5 min at 4 °C. The assay was then performed as directed in the user manual.

SOD Native Gel: The assays were performed with modification as described in a previous report.[20] The KLP lysate was prepared as described above and was then run on a native gel at 40 mA overnight to ensure proper band separation and to reduce smearing. Gels were then negatively stained with a solution of 0.25 mM of nitroblue tetrazolium chloride (NBT), 28 mM TEMED, 0.028 mM riboflavin, and 1 mM EDTA in 50 mM phosphate buffer. The gel was stained for 45 minutes, washed with ddH_2O, soaked in 50 mM phosphate buffer, and exposed to fluorescent light for 15 mins – 2 hours until bands appeared as achromatic on a blue background. The gel was washed with ddH_2O three times and left at room temperature under ambient light for 24 hours to develop further. The gel was then imaged on an Azure Imaging System (Dublin, CA, USA) and processed using ImageJ.

Catalase Native Gel: The bacterial lysate was prepared as described above and run on a native gel as reported.[8c] After running, the gels were washed 3 times for 10 minutes with dH_2O and subsequently incubated in a 0.003% H_2O_2 for 10 minutes. The gels were then washed twice. Gels were then negatively stained with a solution of 2% ferric chloride and 2% potassium ferricyanide in dH_2O. The gel was stained until achromatic bands were formed and then washed extensively with ddH_2O. The gel was then imaged on an Azure Imaging System (Dublin, CA, USA) and processed using ImageJ.

Catalase Activity Assay: The bacterial lysate was prepared as above, and the assay was performed as reported before.[8c] The assay was performed in 50 mM phosphate buffer (pH 7.0) containing 30 mM H_2O_2 and 0.1% triton x-100. The activity was measured as the rate of the conversion of the H_2O_2 to water and oxygen at 240 nm using UV–vis spectroscopy (Agilent 8495, Germany) as previously described.[12a,21] The activity was normalized to the lysate protein concentration analyzed by BCA kit (Pierce BCA protein Assay Kit).

Efficacy in Murine Lung Infection Model: Each group of mice (6 or 3 mice/group depending on the data set) were lightly anesthetized with inhaled isoflurane using a mice anesthesia machine before infection and treatment. KLP was grown in iron-limited BM2 medium at 37 °C, were washed twice in PBS, and resuspended in PBS. Mice were infected intranasally with 8×10^8 CFU/mouse (40 µl inoculum in PBS). After 1 h infection, mice were intranasally administrated with 10 mg /kg of mice GaPP (40 µl), $Ga(NO_3)_3$ (40 µl), or the GaPP/$Ga(NO_3)_3$ combination (40 µl) for 2 days. GaPP stock solution was prepared in DMSO but diluted in PBS before administration. At day 2 post-infection, mice were euthanized with CO_2. Lungs were harvested and homogenized in 1 mL PBS in a tissue homogenizer (Omni International, GA, USA). The CFUs were determined by serial dilution in PBS and culture on tryptic soy agar plates.

Histology: The lungs from mice were extracted and submerged in 4% paraformaldehyde for 24 h of fixation. Paraffin for sectioning, slide fixation, and H&E and Gram staining were conducted by the Tissue Facility of the UNMC. H&E staining was scored for acute pneumonia as 0 = Absent, 1 = Mild, 2 = Moderate and 3 = Severe.

Statistical Analysis: A one-way analysis of variance (GraphPad Prism 8.0) was performed to determine significant differences for multiple comparisons. Percent survival was calculated using GraphPad Prism 8.0. Statistical significance was evaluated at $P < 0.05$, 0.01, 0.001, and 0.0001.

Ethics Statement: Animal studies were performed in a BSL2 room in the Animal Facility of the University of Nebraska Medical Center (UNMC) and conducted according to protocols reviewed and approved by the IACUC # 18-120-09-FC at UNMC. Female BALB/cJ mice aged 8 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Groups of either 3 or 6 mice were housed in individual ventilator cages with unrestricted access to food and water.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

catalase, combination therapy, gallium, iron metabolism, Klebsiella pneumoniae, reactive oxygen species, SOD

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