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Dual Inhibition of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* Iron Metabolism using Gallium Porphyrin and Gallium Nitrate.

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Iron- and heme-uptake pathways and metabolism are promising targets for the development of new antimicrobial agents, as their disruption would lead to nutritional iron starvation and inhibition of bacterial growth. Salts of gallium III (Ga), an iron mimetic metal, disrupt iron-dependent biological processes by binding iron-utilizing proteins and compete with iron for uptake by bacterial siderophore-mediated iron uptake systems. Ga porphyrins, heme mimetic complexes, disrupt heme-utilizing hemeproteins. Because $Ga(NO_3)_3$ and Ga porphyrin disrupt different pathways of bacterial ion acquisition/utilization, we hypothesized that if used in combination they would result in enhanced antimicrobial activity. Antimicrobial activity of Ga porphyrins (Ga protoporphyrin (GaPP) or Ga mesoporphyrin (GaMP)) alone and in combination with $Ga(NO_3)_3$ were evaluated against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and methicillin-resistant *Staphylococcus aureus* (MRSA) under iron-limited conditions. The Ga porphyrin/$Ga(NO_3)_3$ combination demonstrated substantial synergism against *K. pneumoniae*, *P. aeruginosa*, and MRSA. Time-kill assays revealed that the synergistic combination of GaPP/$Ga(NO_3)_3$ was bacteriostatic against *K. pneumoniae* and MRSA and bactericidal against *P. aeruginosa*. The GaPP/$Ga(NO_3)_3$ combination significantly disrupted *K. pneumoniae* and *P. aeruginosa* biofilms on plasma-coated surfaces and increased the survival of *C. elegans* infected with *K. pneumoniae* or *P. aeruginosa*. When assessing the antibacterial activity of the Ga(III)/antibiotic combinations, GaPP or $Ga(NO_3)_3$/colistin combinations also showed synergistic activity against *K. pneumoniae* and *P. aeruginosa*. Our results demonstrate that GaPP and $Ga(NO_3)_3$ have significant synergistic effects against several important human bacterial pathogens by dual inhibition of iron/heme metabolism.

Keywords: Dual inhibition, iron metabolism, Gallium complex, *Pseudomonas aeruginosa*, *Klebsiella pneumonia,* ESKAPE pathogens.

Multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan drug-resistant (PDR) pathogens are limiting the effectiveness of many antibiotic classes. ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) pathogens are among the leading causes of nosocomial infections throughout the world and have developed the resistance to beta-lactam antibiotics by producing beta-lactamase enzymes.[1] *P. aeruginosa* and *K. pneumoniae* are the cause of several potentially lethal infections, including pneumonia, sepsis, wound or surgical site infections, and meningitis. *P. aeruginosa* also causes chronic lung infections in patients with cystic fibrosis (CF) that contribute to the progressive lung failure characteristic of that disease. *P. aeruginosa* and *K. pneumoniae* have intrinsic abilities to develop new mechanisms of resistance and can allow the next generation of bacteria to exhibit drug-resistance as well. Novel antibiotics targeting different pathways are needed. Combinations of drugs that result in synergism is one approach that has been employed against antibiotic resistant bacteria to inhibit both organisms with borderline susceptibility and prevent the emergence of resistance.[2]

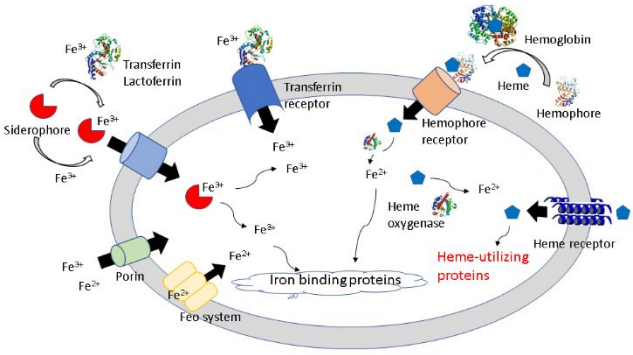

Figure 1. Schematic representation of Iron/Heme acquisition pathways in bacteria. Iron can be transported across bacterial membrane via transferrin, lactoferrin, Feo system, and siderophore-mediated acquisition mechanisms. Heme can be transported via hemophore-mediated transport and direct contact with heme receptors located in the membrane.

Iron acquisition in the human host is critical for bacterial survival.[3] Free iron is sequestered by human host proteins such as transferrin (TF) and lactoferrin (LF), which increase in

concentration upon bacterial invasion, making iron less accessible to bacteria. Many bacterial pathogens combat this iron sequestration through the production of siderophores, low molecular weight compounds that compete for or remove iron from TF and LF.

Many bacterial pathogens have also developed strategies to obtain iron from heme that is tightly complexed with ~70% of iron in the human host (Figure 1).[6] Bacteria acquire heme via either direct contact with exogenous heme or they scavenge heme from host hemoproteins, such as hemoglobin, by releasing hemophores into extracellular space. The exogenous heme and heme-bound hemophores interact with receptors located in membrane, and heme is transported across the membrane into cells, where the heme is degraded by oxygenase and iron is released (Figure 1).

Sequential reduction and oxidation is critical for iron to function at the active site of various enzymes. Gallium (Ga, Fw=69.7) and iron share similar properties, such as ionization potential and electron affinity. Consequently, biologic systems are often unable to distinguish Ga(III) from Fe(III). Ga(III) binds avidly to both TF and LF and is taken up by cells via mechanisms used to acquire iron.[7-9] When Ga(III) replaces iron in iron-centered enzymes, it cannot be reduced to Ga(II), rendering the enzymes non-functional (Figure 1).[14, 15] This allows Ga(III) to interfere with cellular DNA replication by inactivating tumor cell ribonucleotide reductase [16] and has led to its use in treating some cancers and their complications.[17, 18]

Ga-based antimicrobial agents are also being developed to battle drug resistant bacteria.[4, 6, 9, 19-26] Gallium nitrate ($Ga(NO_3)_3$) has been shown to disrupt *P. aeruginosa* and *A. baumannii* iron metabolism and reduced *P. aeruginosa* growth *in vitro*.[27, 28] It also reduced lung bacterial burdens and improved survival when administered to mice.[26-28] *In vitro*, it is bacteriostatic in the

presence of serum or TF and has antimicrobial and anti-biofilm activity.[27, 29] $Ga(NO_3)_3$ targets the *P. aeruginosa* siderophore, pyoverdine, and impacts its production via *pvdS*.[27] $Ga(NO_3)_3$ may inhibit *P. aeruginosa* growth by decreasing the activity of key iron-dependent enzymes, including catalase and ribonucleotide reductase.[21]

Heme, the most abundant iron source available in the human host, plays critical roles in oxygen transport and storage, electron transport, enzymatic reactions, and cellular respiration as a cofactor of hemoproteins in both eukaryotic and prokaryotic cells.[30, 31] A number of studies have demonstrated that non-iron metalloporphyrin analogues, including GaPP, are promising antimicrobial agents.[29, 32-37] Once transported across the bacterial membrane via heme-uptake pathways, GaPP may disrupt functions involving hemeproteins, thereby leading to inhibition of bacterial growth.[35,38]

Many pathogens, including *P. aeruginosa* and *K. pneumoniae* possess and utilize multiple mechanisms of iron acquisition, including siderophore production and heme transport. Since $Ga(NO_3)_3$ inhibits bacterial iron metabolism by targeting siderophore-mediated and free Fe^{3+} uptake pathways and GaPP inhibits the iron metabolism by targeting heme uptake, combination inhibition with these two Ga compounds could lead to enhanced growth inhibition. The presence of both types of gallium compounds would not allow the organism to switch from one iron source to the other as it could in the presence of a single form of gallium. This would enhance antimicrobial activity and potentially reduce the development of resistance. Thus, we tested a combination of two Ga porphyrins, GaPP or GaMP, with $Ga(NO_3)_3$ against *P. aeruginosa*, carbapenem-resistant *K. pneumoniae* (*K. pneumoniae*), as well as two other commonly encountered human pathogens *Acinetobacter baumannii* and *Staphylococcus aureus* (both

methicillin-resistant [MRSA] and methicillin sensitive [MSSA]). In addition, combinations of these gallium compounds with other currently used antibiotics were also investigated for possible synergism.

METHODS

Materials and reagents. Rifampicin (Sigma-Aldrich, St. Louis, MO), amikacin (Fisher Scientific, Fair Lawn, NJ), ceftazidime pentahydrate (Chem-Impex INT'L INC., Wood Dale, IL), levofloxacin (Selleck Chemicals, Houston, TX), colistin sulfate (Research Products International, [RPI], Mount Prospect, IL), minocycline hydrochloride (Sigma-Aldrich, USA), and linezolid (Sigma-Aldrich, USA) were purchased and stored at 0 ~ 4 °C. Gallium(III) protoporphyrin IX chloride (GaPP, >95% purity) and gallium(III) mesoporphyrin chloride (GaMP, >95% purity) were purchased from Frontier Scientific (Logan, UT). Cation-adjusted Mueller-Hinton was from BD (Sparks, MD, USA). Stock solutions were prepared in DMSO for GaPP and GaMP or sterilized water for antibiotics and stored at -20 °C until needed.

Strains. *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* PA103 ATCC 29260, Methicillin-Susceptible *Staphylococcus aureus* (MSSA) ATCC 25923 and cabapenemase-resistant *Klebsiella pneumoniae* ATCC BAA 1705 (*K. pneumoniae*) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Colistin-resistant *P. aeruginosa* and Methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained from the Clinical Pathology/Microbiology Laboratory at Nebraska Medicine, Omaha, NE. *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* were cultured in iron-depleted BM2 medium (pH 7.0) prepared from potassium phosphate dibasic (6.97g/L, EMD, Germany), potassium phosphate monobasic (2.99g/L, EMD, Germany), ammonium sulfate (0.92g/L, Sigma-Aldrich, USA), magnesium sulfate $7H_2O$ (0.24g/L, Sigma-Aldrich, USA), succinic acid (4.02g/L, Sigma-

Aldrich, USA) and casamino acid (1g/L, Difco, USA).[39] MRSA (USA300) and MSSA were cultured in cation-adjusted Mueller-Hinton (CAMH, 3g/L).

Minimum Inhibitory Concentration. Antimicrobial susceptibility testing was performed using two serial microdilution methods in a 96-well plate. *P. aeruginosa* PA103 (OD_{625} = 0.4, 6.3 x 10^8 CFU/mL), *K. pneumoniae* (OD_{600} = 0.4, 5.0 x 10^8 CFU/mL), and *A. baumannii* (OD_{600} = 0.4, 8.5 x 10^7 CFU/mL) were cultured overnight in BM2. Each well of the plate was inoculated with 5 x 10^5 CFU/mL and incubated in BM2 at 37° C for 24 h. MRSA USA300 (OD_{600} = 0.1, 1 x 10^8) and MSSA (McFarland = 0.5, 1.5 x 10^8 CFU/mL) were grown in iron-poor CAMH and diluted to inoculate each well of the 96-well plate with 1 x 10^6 CFU/mL. OD_{600} was measured to determine the lowest concentration of the compound resulting in growth inhibition using a Biotek Synergy H1 hybrid Reader.

Checkerboard assay. The microdilution method was performed to determine a synergistic, indifferent, or antagonistic effect of the combination of antibiotics. The first compound of the combination was serially diluted from the bottom to the top of the plate ranging from 0 to MIC, while the other antibiotic was diluted from the left to the right ranging from 0 to MIC. For three-dimensional assay, the concentration of an antibiotic was fixed in 7 plates, ranging from 0 to MIC, while the concentration of the Ga compounds was increased from 0 to MIC on each x and y axis of the plates. Each microtiter well was inoculated with a bacterial inoculum of 5 × 10^5 CFU/ml or 1 × 10^6 CFU/mL, as described above. The plates were incubated at 37°C for 24 h (Gram-negative bacteria) or 48 h (MRSA) under aerobic conditions. OD_{600} was measured to determine synergistic effects of the combinations of two and three antibiotics. The fractional inhibitory concentration index (FICI) was then calculated. The ΣFICIs were then calculated as follows: ΣFICI = FICI A + FICI B or ΣFICI = FICI A + FICI B + FICI C. FICI A is

$MIC_{A(combination)}/MIC_A$, FICI B is $MIC_{B(combination)}/MIC_B$, and FICI C is $MIC_{C(combination)}/MIC_C$ in the combination, where MIC_{ABC} is the lowest concentration of the combination that inhibits the bacterial growth and MIC_A, MIC_B, and MIC_C are the lowest concentrations of drug A, B, and C alone in the combination. A synergistic effect is considered present when the ΣFICI is ≤ 0.5. The ΣFICI of > 0.5 and the ΣFICI of ≥ 2 are considered indifferent and antagonistic, respectively.

Time-kill assay. Time-kill studies were performed to compare the time required to kill bacteria by individual antibiotics and when they were combined with other agents. In brief, 1×10^6 CFU/mL mid-log-phase *K. pneumoniae* and *P. aeruginosa* PA103 were incubated in BM2 containing GaPP plus $Ga(NO_3)_3$ or alone in a total volume of 4 mL at 37° C. Samples of 0.05 mL were obtained at multiple time points up to 24 h, and then plated on tryptic agar plates for counting of colony forming units (CFU). Assays were performed in triplicate.

Biofilm assay. The microtiter plate assay for biofilm formation was performed according to a reported method with modification.[40] Briefly, a tissue culture 96 well plate (Corning, NY, USA) was precoated with 20% human plasma (Sigma) in 0.05 M carbonate buffer, pH 9.4 at 4° C for 24 h. After decanting the buffer, overnight cultures of *P. aeruginosa* PA103 and *K. pneumoniae* in BM2 were diluted to OD_{600} = 0.05 and used to form biofilm in TSB medium supplemented with 3% NaCl, 0.5% casamino acids, and 0.5% glucose. The plate was incubated at 37° C for 24 h and washed with PBS buffer, following which BM2 containing gallium porphyrin, gallium nitrate or a combination was added to each well in triplicate. After 24 h of incubation at 37° C, the biofilm was scraped into PBS buffer (100 μL), serially diluted, and plated on TSA plates to determine CFU.

***Caenorhabditis elegans* killing assay**. Wild type N2 *C. elegans* was purchased from the Caenorhabditis Genetics Center at the University of Minnesota and cultured as described.[41, 42] Synchronized L1 stage worms were allowed to grow to L4 stage/young adult on Nematode Growth Medium (NGM) agar plate seeded with *E. coli* OP50. *C. elegans* were collected and washed with sterilized M9 buffer three times by centrifugation at 100 x g for 1 min. *C. elegans* were resuspended in a M9:slow killing (2:1) medium. 20~30 *C. elegans* were transferred to 96-well plates containing Ga(III) compounds and infected with *P. aeruginosa* PA103 or *K. pneumoniae* for 17 ~ 24 hour at 23° C. After gentle shaking of the plate by hand, the worms were considered to be dead if they did not move.

Statistical Analysis. A one or two-way analysis of variance (ANOVA, Turkey, Graphpad prism 7.0) was performed to determine significant differences for multiple comparison. Student's t-test was performed to compare two groups. Statistical significance was evaluated at $P < 0.05$, 0.01 and 0.001.

Table 1. Minimum inhibitory concentrations of gallium protoporphyrin, gallium mesoporphyrin, gallium nitrate, and other antibiotics.

	MIC (μg/mL)									
	$Ga(NO_3)_3$	GaMP	GaPP	CO	CZ	AK	LF	RF	LZ	MC
P. aeruginosa[a]	2	-	8	16	-	-	-	-	-	-
P. aeruginosa	1	8	8	1	1	16	0.5	8	-	-
K. pneumoniae	4	16	16	64	16	256	128	8	-	-
A. baumannii	4	4	4	8	16	32	0.25	0.5	-	-
MRSA USA300	512	0.5	0.05	-	-	-	-	0.012	1	0.25
MRSA LAC JE2	512	0.5	0.062	-	-	-	-	0.012	2	0.125
MSSA	512	0.25	0.031	-	-	-	-	0.012	2	0.125

(a) Colistin-resistant clinical isolate. Media; BM2 for *P. aeruginosa* and *K. pneumoniae* ATCC 1705 (*K. pneumoniae*), CAMH (3g/L) for *S. aureus*. CO: colistin; CZ: ceftazidime; AK: amikacin; LF: levofloxacin; RF: rifampicin; LZ: linezolid; MC: minocycline. (-) not determined.

RESULTS

Minimum inhibitory concentration. The MICs of GaPP, GaMP, and $Ga(NO_3)_3$ were determined for several Gram-positive and Gram-negative bacterial species (Table 1). All Gram-

negative bacteria were cultured in iron-depleted BM2 medium (see method for preparation). MRSA and MSSA strains were cultured in 3g/L CAMH since they did not grow very well in BM2. The antimicrobial activity of Ga(III) compounds was compared to 4 antibiotics against the Gram negative bacteria - colistin sulfate, ceftazidime pentahydrate, amikacin, and levofloxacin, reflecting four different antibiotic classes - polypeptide, β-lactam, aminoglycoside, and fluoroquinolone, respectively. Here, *K. pneumoniae* was found to be resistant to colistin, amikacin, and levofloxacin, exhibiting MICs of >64 μg/mL in iron-depleted medium. MICs of three antibiotics (rifampicin, minocycline, and linezolid) were tested against *S. aureus* strains. The antibiotics are active against all *S. aureus* with similar MICs (Table 1). In contrast, none of the *S. aureus* strains were susceptible to $Ga(NO_3)_3$, exhibiting MICs of 512 μg/mL in iron-poor CAMH (3g/L) (Table 1). The non-susceptibility of *S. aureus* strains suggests that the antimicrobial efficacy of $Ga(NO_3)_3$ is influenced by the presence of iron. The MICs for other pathogens ranged from 1–4 μg/mL, indicating that these pathogens are susceptible to $Ga(NO_3)_3$ in iron-limited conditions.

All pathogens tested in our study were susceptible to Ga porphyrin under iron-limited conditions. Ga porphyrin (GaPP and GaMP) treatment led to significant growth inhibition, with MIC values of ≤ 1 μg/mL against *S. aureus* strains (Table 1). The MICs of GaMP and GaPP were 0.25-0.5 μg/mL and 31-62 ng/mL, respectively, against *S. aureus* strains (MRSA and MSSA). Interestingly, the MICs of GaMP were ≥8 times higher than those of GaPP against the *S. aureus* strains, which was not observed with the other pathogens. Among the Gram-negative bacteria, carbapenem-resistant *K. pneumoniae* was less susceptible to GaMP and GaPP, with MICs of 16 μg/mL.

Table 2. The lowest fractional inhibitory concentration index (FICI) for combinations.

	FICI (μg/mL)			
Combination	*P. aeruginosa*	*K. pneumoniae*	*A. baumannii*	MRSA
GaPP/$Ga(NO_3)_3$	0.50 (0.004/0.5)	0.13 (0.125/1)	0.50 (1/1)	0.37 (0.0125/64)
GaMP/$Ga(NO_3)_3$	0.50 (0.004/0.5)	0.13 (0.125/1)	0.53 (2/0.12)	0.25 (0.062/64)
GaPP/CO	0.50 (0.031/0.5)	0.25 (2/8)	0.37 (1/1)	-
GaPP/LF	1.00 (0.03/0.5)	0.50 (0.03/64)	1.00 (4/0.125)	-
GaPP/CZ	1.00 (0.03/1)	0.62 (2/8)	1.00 (4/0.25)	-
GaPP/AK	1.00 (0.03/16)	1.00 (0.03/256)	1.00 (4/0.125)	-
$Ga(NO_3)_3$/CO	0.50 (0.002/0.5)	0.37 (0.5/16)	-	-
$Ga(NO_3)_3$/LF	0.53 (0.125/0.125)	1.00 (0.03/64)	-	-
$Ga(NO_3)_3$/CZ	0.75 (0.5/0.5)	0.75 (1/8)	-	-
$Ga(NO_3)_3$/AK	1.00 (2/0.062)	1.00 (0.03/128)	-	-
GaPP/RF	0.25 (0.031/2)	1>	-	0.25 (0.195/6.25)[a]
GaPP/LZ	-	-	-	0.50 (0.006/0.5)
GaPP/MC	-	-	-	0.37 (12.5/31.2)[a]

(a) ng/mL, FIC index: Synergy (≤ 0.50), additivity or indifference (0.50 < FICI ≤ 2), antagonism (> 2). CO: colistin; CZ: ceftazidime; AK: amikacin; LF: levofloxacin; RF: rifampicin; LZ: linezolid; MC: minocycline.

Checkerboard assay. To determine potential synergistic antimicrobial efficacy of the combination of GaPP and $Ga(NO_3)_3$, or a panel of currently available antibiotics, checkerboard assays were performed and the fractional inhibitory concentration index (FICI) was calculated (Table **2**). With the combination of $Ga(NO_3)_3$ and GaPP or GaMP, the most synergistic effect was observed against *K. pneumoniae* (FICI = 0.13). Against *P. aeruginosa* the gallium porphyrin/$Ga(NO_3)_3$ combination also showed a synergistic effect, with FICI = 0.50 at concentrations of 4 ng/mL GaPP or GaMP and 0.5 μg/mL $Ga(NO_3)_3$. Interestingly, $Ga(NO_3)_3$ showed one-fold higher activity while GaPP or GaMP concentration was substantially reduced to 4 ng/mL from its MIC in Ga porphyrin/$Ga(NO_3)_3$ combination (Figure S1). In addition, the GaPP/$Ga(NO_3)_3$ combination demonstrated a synergistic activity against *A. baumannii* (FICI = 0.50). The GaPP or GaMP/$Ga(NO_3)_3$ combination also demonstrated a synergistic antimicrobial activity against a MRSA USA300 (FICI = 0.37 and 0.25, respectively). The MICs of GaPP and

GaMP against this isolate was reduced in the presence of $Ga(NO_3)_3$. In this study with Ga porphyrin/$Ga(NO_3)_3$ combinations, no antagonistic activity was found against any tested bacteria.

We also investigated the potential for synergistic antimicrobial efficacy of $Ga(NO_3)_3$ in combination with other antibiotics against *P. aeruginosa* and *K. pneumoniae* and found synergistic effects for the $Ga(NO_3)_3$/colistin combination with FICIs of 0.50 and 0.37, respectively. Similarly, synergistic effects were demonstrated with GaPP/colistin (FICI = 0.25) and GaPP/levofloxacin (FICI = 0.50) combinations against *K. pneumoniae* and GaPP/colistin (FICI = 0.50) and GaPP/rifampin (FICI = 0.25) against *P. aeruginosa*.

For Gram-positive bacteria, we chose rifampicin, linezolid, and minocycline, which are widely used to treat MRSA infections, to investigate synergistic effects in combination with GaPP against MRSA. Evaluation of antibacterial effects of GaPP in combination with rifampicin, linezolid or minocycline revealed synergistic activity with FICI values of 0.25, 0.5, and 0.37, respectively, against MRSA USA300 (Table **2**).

Table 3. The FICI values for the GaPP/$Ga(NO_3)_3$/colistin combination.

		GaPP/$Ga(NO_3)_3$/Colistin (μg/mL)				
K. pneumoniae	Conc.	2/0.25/4	4/0.25/4	2/0.25/8	2/0.25/16	2/0.5/4
	FICI	0.22	0.25	0.28	0.41	0.25
P. aeruginosa	Conc.	0.03/0.25/0.12	0.06/0.25/0.12	0.12/0.25/0.12	0.25/0.25/0.12	0.5/0.25/0.12
	FICI	0.38	0.38	0.39	0.41	0.44

FIC index: Synergy (≤ 0.50), additivity or indifference (0.50 < FICI ≤ 2), antagonism (> 2).

Among tested bacteria, *K. pneumoniae* and *P. aeruginosa* were susceptible to both GaPP/$Ga(NO_3)_3$ and Ga(III)/colistin combinations. Thus, we investigated the activity of three-drug combination in the inhibition of both pathogens (Table 3). Synergism of the

GaPP/$Ga(NO_3)_3$/colistin combination was found for *K. pneumoniae* and *P. aeruginosa* with FICI values ranging from 0.22 to 0.50. Although no significant reduction of $FICI_{GaPP/Ga(NO_3)3/colistin}$ compared to FICIs of two-Ga(III) combinations was observed, the addition of colistin to the GaPP/$Ga(NO_3)_3$ combination exhibited significant antimicrobial activity against both pathogens.

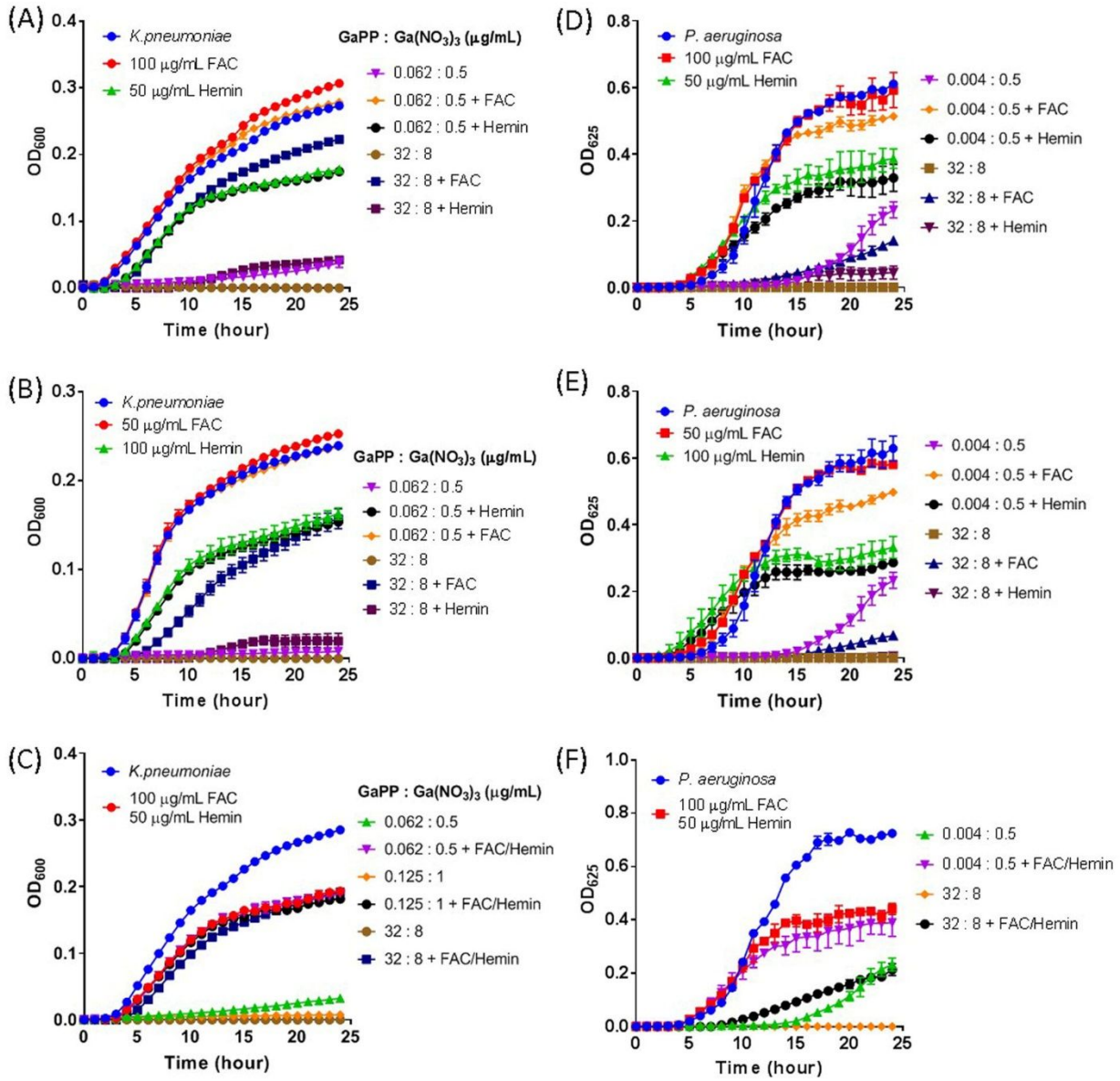

Figure 2. Growth curve and recovery of *K. pneumoniae* (A-C) and *P. aeruginosa* (D-F) inhibited by a GaPP/$Ga(NO_3)_3$ combination in the presence of iron sources. The growth curves were obtained by measuring OD_{600} or OD_{625}. FAC: ferric ammonium citrate. Data are mean ± standard deviation of triplicate experiments.

Growth recovery of *K. pneumoniae* and *P. aeruginosa* inhibited by GaPP/$Ga(NO_3)_3$ combinations in the presence of FAC, hemin, or both. It was expected that $Ga(NO_3)_3$ and Ga porphyrins were working by disrupting different iron acquisition pathways. To test this hypothesis, we investigated whether exogenous iron and hemin would increase the growth of *K. pneumoniae* and *P. aeruginosa* in iron-depleted BM2. At First, experiments were performed with *K. pneumoniae*, since Ga porphyrin/$Ga(NO_3)_3$ combinations demonstrated the lowest FIC index against this bacteria (Table 2), allowing for enhanced ability to detect an effect. In the absence of Ga(III), the addition of FAC (50 and 100 μg/ml) did not significantly increase the *K. pneumoniae* growth rate, showing only 6 and 12% increases in the population, respectively, at 24 h compared to non FAC/hemin-supplemented control (Figure 2A and 2B). In contrast, 32-35% growth reduction was observed at 24 h compared to (+) control when with the addition of hemin (50 and 100 μg/mL), which indicates hemin toxicity (Figure 2) Interestingly, , the growth of *K. pneumoniae* treated with both FAC and hemin was reduced by 32% compared to (+) control at 24 h (Figure 2C and Figure S7). Thus, FAC does not reverse *K. pneumoniae* growth inhibition by excess hemin.

Although some reversal of growth inhibition of *K. pneumoniae* by a series of GaPP/$Ga(NO_3)_3$ combinations was observed with the addition of either FAC or hemin, FAC was more effective as exogenous iron source (Figure 2). The addition of FAC rescued the *K. pneumoniae* growth by 73-91% and 62-95% at concentrations ranging from 0.062/0.5 to 32/8 μg/mL (GaPP/$Ga(NO_3)_3$) compared to 100 and 50 μg/mL FAC alone treated controls, respectively. As expected, addition of hemin inhibited the bacterial growth by 40% compared to (+) control (Figure 2A and 2B). Compared to 100 and 50 μg/mL hemin treated controls, 24-98% and 15-94% growth recovery was observed at the same concentration range (0.062/0.5 to 32/8 μg/mL) of the GaPP/$Ga(NO_3)_3$

combinations, respectively (Figure 2A and 2B). However, the ability of FAC or hemin to produce growth recovery declined at higher concentrations of the Ga inhibitors. At 32/8 μg/mL GaPP/$Ga(NO_3)_3$, hemin rescued the growth only 8-12% compared to hemin-treated controls at 24 h of incubation (Figure 2A, 2B, and Figure S7). Interestingly the addition of both FAC and hemin in combination did not decrease the growth recovery with increasing the concentration (Figure S7F).

We also studied the growth recovery of *P. aeruginosa* in the presence of FAC and hemin (Figure 2) and found a similar pattern to that of *K. pneumoniae* growth recovery. Excess FAC did not promote *P. aeruginosa* growth rate significantly, but growth inhibition was reversed with the addition of an exogenous iron source. At 32/8 μg/mL GaPP/$Ga(NO_3)_3$, excess hemin and FAC rescued the growth only 8-12% compared to hemin-treated controls at 24 h of incubation (Figure 2D and 2E). This may be due to excess hemin, which resulted in toxicity as manifested by a reduction of growth rate. The results suggest that the combination of GaPP and $Ga(NO_3)_3$ has high potential for development as an antimicrobial agent against both *K. pneumoniae* and *P. aeruginosa*.

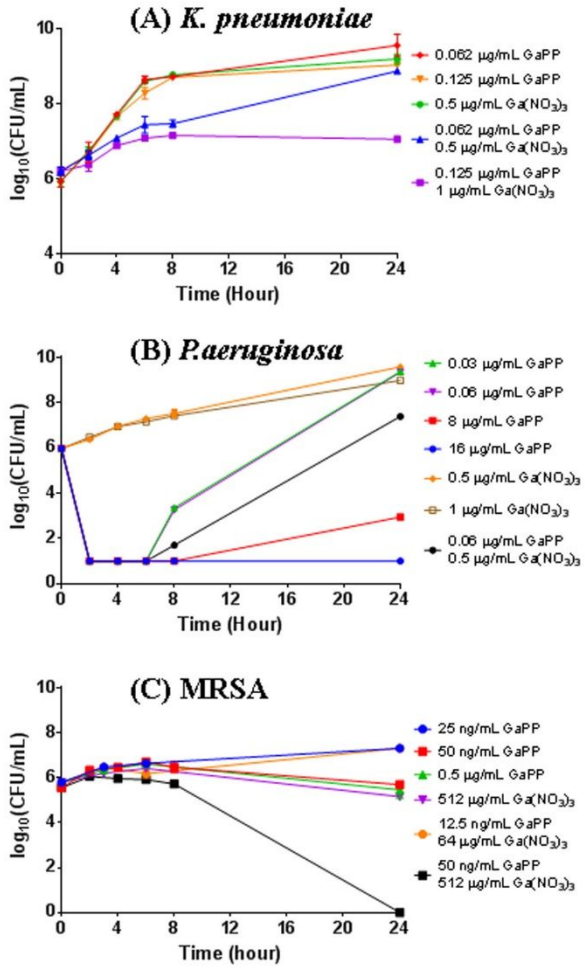

Figure 3. Time-kill assay of combinations of GaPP and $Ga(NO_3)_3$ against (A) *K. pneumoniae*, (B) *P. aeruginosa*, and (C) MRSA USA 300. *K. pneumoniae* and *P. aeruginosa* were cultured in iron-depleted BM2 and MRSA was cultured in CAMH (3g/L) for 24 h. Data are the mean ± standard deviation of triplicate experiments.

Time-Kill assay. Time-kill assays were performed to further investigate the activities of the GaPP/$Ga(NO_3)_3$ combinations in iron-poor conditions (Figure 3). For the time-kill assay, the lowest concentrations of compounds that exhibited best activity were chosen from the checkerboard assay results. Growth inhibition varied with bacterial species (*K. pneumoniae*, *P. aeruginosa* and MRSA USA300). The most effective synergy was found against *K. pneumoniae* at the combination of GaPP (0.125 μg/mL, 0.008 x MIC) and $Ga(NO_3)_3$ (1 μg/mL, 0.125 x MIC) with bacteriostatic activity, whereas GaPP or $Ga(NO_3)_3$ alone did not achieve growth inhibition at this concentration, resulting in a >2.5 log growth increase (Figure 3A).

In contrast to the bacteriostatic growth inhibition observed in the time-kill studies against *K. pneumoniae*, higher concentrations of GaPP (8 and 16 μg/mL) showed bactericidal activity against *P. aeruginosa* with > 3 and 5 log reductions, respectively, at 1 x 10^6 CFU/mL inoculum (Figure 3B). The lower concentrations of GaPP (0.03 and 0.06 μg/mL) also exhibited bactericidal activity by 6 h, but regrowth was observed by 24 h of incubation. At 0.06 μg/mL (0.008 x MIC)/0.5 μg/mL (0.5 x MIC), the GaPP/$Ga(NO_3)_3$ combination led to kill *P. aeruginosa* with > 5 log reductions within 6-8 h, but regrowth was observed and resulted in ~1.5 log increase at 24 h of incubation (Figure 3B). In order to determine stable resistance to the inhibitor combination, two independent time-kill assays were performed (Figure S8). Addition of GaPP/$Ga(NO_3)_3$ (0.06/0.5 μg/mL) at 6 h or 24 h of incubation did not completely inhibit the regrowth of *P. aeruginosa* (Figure S8A). A time-kill assay with regrown cells at 1 x 10^6 CFU/mL inoculum indicated resistance to the combination inhibitor (Figure S8B).

The combination of GaPP (50 ng/mL, 1 x MIC) and $Ga(NO_3)_3$ (512 μg/mL, 1 x MIC) achieved complete killing of MRSA, thereby showing the most effective and synergistic bactericidal effect (Figure 3C). However, the combination of 12.5 ng/mL GaPP (0.25 x MIC) and 32 μg/mL $Ga(NO_3)_3$ (0.06 x MIC) exhibited a bacteriostatic effect, with about a 1 log increase of the MRSA population in 24 h (Figure 3C). This bacteriostatic effect was also observed when MRSA was exposed to 0.5 x MIC of GaPP alone or 1 x MIC of $Ga(NO_3)_3$ alone. Interestingly, there was no significant difference between a 10 x MIC (0.5 μg/mL) and 1 x MIC GaPP in the inhibition of MRSA growth in iron-poor medium (3g/L CAMH).

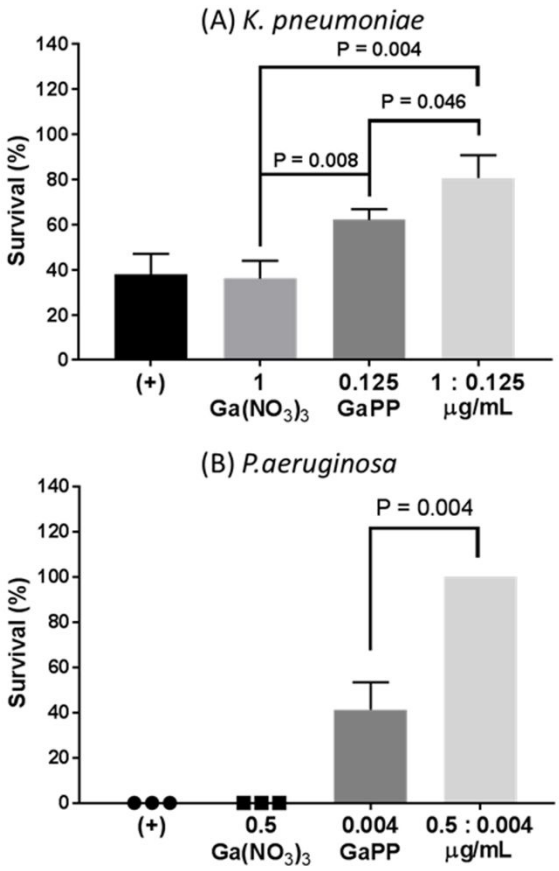

Figure 4. Anti-biofilm activity of GaPP, $Ga(NO_3)_3$, and combination. Biofilms were formed on plasma-coated plates. (A)*K. pneumoniae*, (B) *P. aeruginosa*. Significance was calculated using Student's T test compared to (+) control. Data are the mean ± standard deviation of triplicate experiments.

Substantial disruption of *K. pneumoniae* or *P. aeruginosa* biofilms by GaPP in combination with $Ga(NO_3)_3$. The checkerboard assay demonstrated that the GaPP/$Ga(NO_3)_3$ combination exerted the most effective synergistic antimicrobial activity against *K. pneumoniae* and *P. aeruginosa*. Thus, anti-biofilm activity of GaPP/$Ga(NO_3)_3$ combinations was investigated against *K. pneumoniae* and *P. aeruginosa*. The lowest concentrations of gallium compounds that display complete inhibition in checkerboard assays were chosen for their ability to disrupt biofilms grown on plasma-coated plates (Table 2). CFU determination for the biofilm disruption by combination inhibition indicated exceptional biofilm reduction when compared to the non-treated control, while both GaPP and $Ga(NO_3)_3$ alone exhibited no significant effect on either

pathogen biofilm (Figure 4). A GaPP (125 ng/mL) in combination with $Ga(NO_3)_3$ (1 μg/mL) disrupted > 90% *K. pneumoniae* biofilm ($P < 0.001$), and a GaPP (4 ng/mL)/$Ga(NO_3)_3$ (0.5 μg/mL) combination showed > 95% disruption ($P < 0.01$) in *P. aeruginosa* biofilm compared to their respective non-treated control.

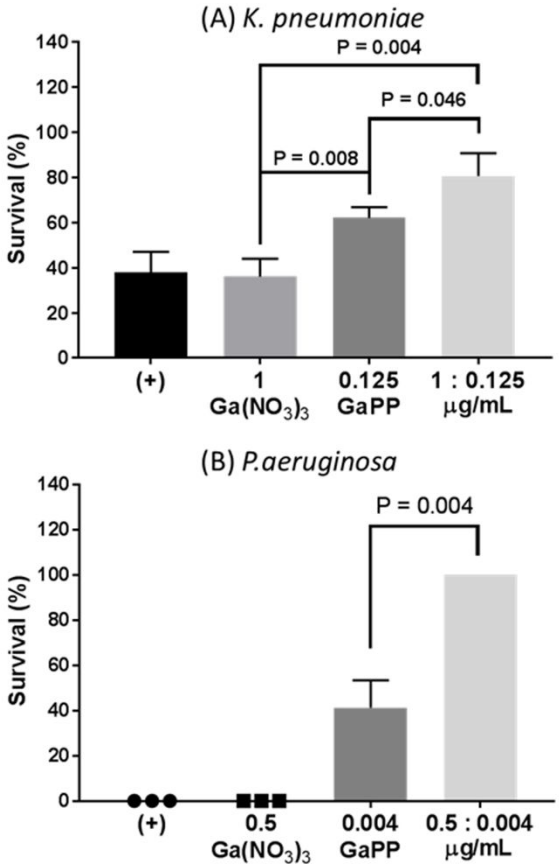

Figure 5. *In vivo* antimicrobial activity of GaPP, $Ga(NO_3)_3$, and a combination of the two on *K. pneumoniae*- and *P. aeruginosa*-infected *C. elegans*. Significance was calculated using Student's T test compared to (+) control. Data are the mean ± standard deviation of triplicate experiments.

A GaPP/$Ga(NO_3)_3$ combination against C. *elegans* infected with *K. pneumoniae* or *P. aeruginosa*. We tested the GaPP/$Ga(NO_3)_3$ combination for its ability to rescue *C. elegans* infected with *K. pneumoniae* or *P. aeruginosa* under liquid media conditions. To test the effect of combination inhibitor, we chose concentrations of GaPP/$Ga(NO_3)_3$ that inhibit the growth of

each pathogen with the lowest FIC index (Table 2). As shown in Figure 5, hermaphrodite *C. elegans* (L4 - young adult) died over a period of 24 h when feeding in M9 media containing *P. aeruginosa* while 38% of *K. pneumoniae*-infected *C. elegans* survived, indicating that *P. aeruginosa* is more lethal to *C. elegans*. $Ga(NO_3)_3$ alone did not show antimicrobial activity against the lethality of either pathogen. However, 25 and 38% survival of *C. elegans* was observed with *K. pneumoniae* and *P. aeruginosa* when exposed to 125 and 4 ng/mL GaPP alone, respectively, compared to their (+) controls. Combination inhibition was much more efficacious than mono-inhibition. There was 100% survival of *P. aeruginosa*-fed *C. elegans* by the treatment of 0.004/0.5 μg/mL GaPP/$Ga(NO_3)_3$ and 80% survival of *K. pneumoniae*-fed *C. elegans* by the treatment of 0.125/1 μg/mL GaPP/$Ga(NO_3)_3$.

DISCUSSION

Non-iron metalloporphyrins are potential antimicrobial agents with broad-spectrum activity against Gram-negative and Gram-positive pathogens and mycobacteria. It has been shown that these porphyrins are taken up via heme-uptake pathways by bacteria, thereby disrupting heme/iron utilizing mechanisms.[43] GaPP and GaMP are mimics of natural heme and demonstrated antimicrobial activity against several pathogens including MRSA,[44] *P. aeruginosa*,[35, 45] *A. baumannii*,[33] *M. abscessus*,[46] and sexually transmitted pathogens.[32] $Ga(NO_3)_3$, FDA-approved for hypercalcemia of malignancy, has also been investigated as antimicrobial agent against several pathogens.[11, 20, 28, 47-49] A recent study reported that systemic Ga treatment improved lung function in people with cystic fibrosis and chronic *P. aeruginosa* lung infections in a preliminary phase 1 study.[50]

Given their potential for disrupting different aspects of bacterial iron acquisition, we investigated whether GaPP or GaMP in combination with $Ga(NO_3)_3$ as well as other antibiotics demonstrate synergistic activity against several human pathogens. In general, GaPP/$Ga(NO_3)_3$ or GaMP/$Ga(NO_3)_3$ combinations demonstrated synergistic or indifferent effect against all pathogens tested in our study. Combinations of Ga porphyrin and $Ga(NO_3)_3$ exhibited the best synergistic activity against *K. pneumoniae* with FICI = 0.13 (Table 2). GaPP and GaMP also synergized with $Ga(NO_3)_3$ with FICI = 0.5 in growth inhibition of *P. aeruginosa*. Interestingly, concentrations as low as 4 ng/mL GaPP alone, 2000 times lower than MIC, still inhibited about ~ 30% of *P. aeruginosa* growth in iron-depleted medium (Figure S1A). This partial inhibition may lower the $Ga(NO_3)_3$ concentration that exerts synergistic killing in combination with GaPP. Consistently, the synergistic activity of the Ga porphyrin and $Ga(NO_3)_3$ combination was also observed against MRSA. These *in vitro* susceptibility data suggest that Ga porphyrin in combination with $Ga(NO_3)_3$ may be advantageous to treat infections caused by pathogens tested in our study.

We also selected four antibiotics (colistin, amikacin, levofloxacin, and ceftazidime) which are being used to treat infections caused by Gram-negative pathogens for our combination studies with GaPP or $Ga(NO_3)_3$ and investigated synergism against the Gram-negative bacteria *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. In checkerboard assays (Table 2), FICI values revealed that GaPP/antibiotic combinations did not exert synergism, but rather indifference, against Gram-negative pathogens tested in our study. The exception was the GaPP/colistin combination against *K. pneumoniae* (FICI = 0.25), *P. aeruginosa* (FICI = 0.50), and *A. baumannii* (FICI = 0.37). Likewise, the $Ga(NO_3)_3$/colistin combination demonstrated FICIs of 0.50 and 0.37 against *P. aeruginosa* and *K. pneumoniae*, respectively, which are in agreement

with other studies. Synergistic effects of the $Ga(NO_3)_3$/colistin combination were reported against multi-drug resistant *A. baumannii*, with a significant reduction of MICs of colistin in the presence of $Ga(NO_3)_3$.[28] Also, Goss et al., reported that $Ga(NO_3)_3$ has synergistic effect with colistin against *P. aeruginosa* PAO1.[21] Our results also showed synergistic killing of *K. pneumoniae* and *P. aeruginosa* by a GaPP/$Ga(NO_3)_3$/colistin combination.

MIC values in Table 1 suggested that *S. aureus* is highly susceptible to GaPP, implying efficient heme-uptake mechanism. Also, GaPP is 10-fold more active than GaMP against *S. aureus* and may be due to different physical property however in-depth studies are ongoing. Checkerboard assays revealed that the addition of rifampin, linezolid, or minocycline to GaPP produced synergistic responses against MRSA (Table 2). The best synergistic killing (FICI = 0.25) was found in the combination of GaPP and rifampin. Rifampin, a semisynthetic derivative of rifamycin, shows bactericidal effects against MRSA by inhibiting DNA-dependent RNA polymerase. However, it has been observed that bacteria quickly develop resistance to rifampin monotherapy, suggesting a use of rifampin with another antimicrobial agent(s) to prevent the emergence of rifampicin-resistant bacteria.[51, 52] Thus, our results suggest that the emergence of resistance to rifampin might be prevented by the addition of GaPP.

There are several different testing methods, including checkerboard assay, time-kill assay, ETEST, and multiple-combination bactericidal test to determine synergism *in vitro*.[53] Although checkerboard testing is among the most widely used technique, it is necessary to perform another combination technique to compare the consistency of the results, which might provide better correlation with the clinical synergistic testing. We thus performed time-kill assays against *K. pneumoniae*, *P. aeruginosa*, and MRSA for GaPP/$Ga(NO_3)_3$ combinations (Figure 3). GaPP

alone or in combination with GaPP/$Ga(NO_3)_3$ displayed bacterial species-dependent bactericidal activity in the *in vitro* time kill assay. A GaPP/$Ga(NO_3)_3$ (0.125/1 μg/mL, 0.008 x MIC/ 0.125 x MIC) combination exerted bacteriostatic activity against *K. pneumoniae* while the GaPP/$Ga(NO_3)_3$ combination achieved bactericidal killing at concentrations of 0.062/0.5 at 6 h of incubation against *P. aeruginosa*. Moreover, bactericidal activity was observed with GaPP mono-inhibition at concentrations of 0.031 and 0.062 μg/mL against *P. aeruginosa* at 6 h of incubation, even though regrowth was observed at 24 h. Complete killing of *P. aeruginosa* with GaPP mono-inhibition at 2 x MIC indicates that *P. aeruginosa* could be more sensitive to GaPP due to more efficient heme-uptake. Interestingly, gallium porphyrins, GaPP and GaMP, showed significant growth inhibition of MRSA strains, with MICs of ≤ 50 and ≤ 400 ng/mL under iron-poor medium, respectively (Table 1). The nanogram ranges of MICs are in agreement with other reports suggesting that *S. aureus* may have more active heme-acquisition systems than iron-acquisition systems such as siderophores.[54] Increased GaPP susceptibility was also observed against bacteria possessing extra heme-uptake gene cluster (*hemO*) in addition to the *hemT* gene cluster. *A. baumannii* ACICU, which possesses both heme-uptake gene clusters, showed high sensitivity to GaPP compared to other strains with only the *hemO* cluster when cultured in medium containing albumin that is known to bind to GaPP.[36] Time-kill studies demonstrated that treatment of GaPP or $Ga(NO_3)_3$ mono-inhibition at their MICs had a bacteriostatic effect against MRSA USA300. A synergistic combination of compounds should lower concentrations of antibiotics in combination, which decreases the incidence of emergence of multi-drug resistant bacteria and reduce drug's toxicity. The GaPP/$Ga(NO_3)_3$ combination at 0.0125/64 μg/mL showed synergism against MRSA, but bacteriostatic. On the contrary, the combination of GaPP and $Ga(NO_3)_3$ at their individual MICs exhibited bactericidal activity against MRSA, presumably

by disrupting both iron and heme metabolisms at concentrations of 0.05/512 μg/mL (GaPP/$Ga(NO_3)_3$).

A similar study was recently reported that the combination of the iron-chelator deferiprone and GaPP showed synergistic effects against *S. aureus*, its biofilm, and biofilm-associated small colony variants, demonstrating concentration- and strain-dependent antibacterial and anti-biofilm activities *in vitro* and *in vivo*.[55] As shown in Figure 4 and 5, the combination of GaPP and $Ga(NO_3)_3$ exhibited significant anti-biofilm against *K. pneumoniae* and *P. aeruginosa*, as well as antimicrobial activity against *C. elegans* infected with either pathogen. Biofilm-forming bacteria can produce a thick layer of extracellular biofilm as a virulence factor that protect microorganisms from defensive immune systems and the effects of antimicrobial agents. Bacteria in biofilms are considerably more resistant to antibiotics than planktonic cells. Therefore, treatment of an infection after a biofilm has been formed is frequently futile with the current inhibition options. Previous work demonstrated that $Ga(NO_3)_3$ inhibits biofilms formed by *P. aeruginosa.*[27] In our study, biofilms were prepared on plasma-coated plates for determination of anti-biofilm activity. We found that the GaPP/$Ga(NO_3)_3$ combination remarkably reduces bacterial populations in *K. pneumoniae* and *P. aeruginosa* biofilms, exerting astonishing synergistic anti-biofilm activity. Our findings suggest that the GaPP/$Ga(NO_3)_3$ combination is a promising therapeutic strategy to combat *K. pneumoniae* and *P. aeruginosa* biofilm infections.

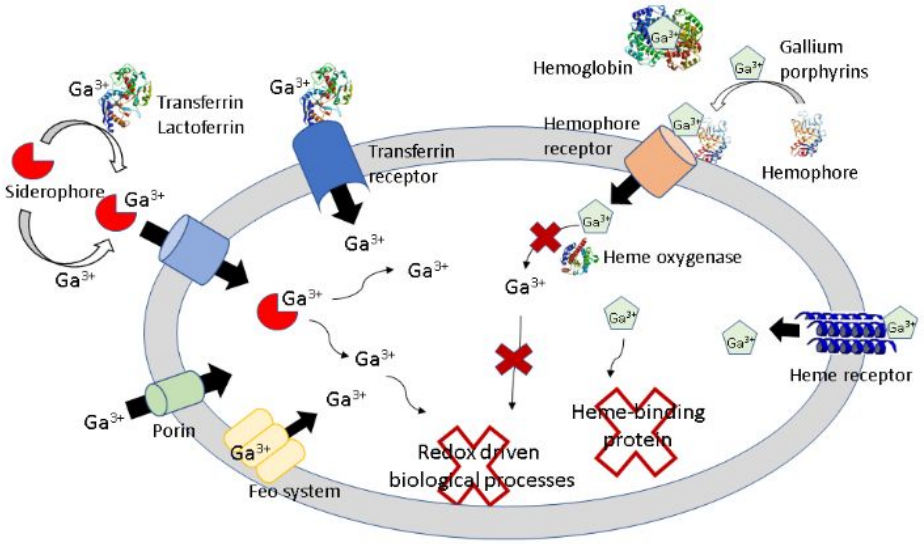

Figure 6. Schematic representation of gallium uptake and disruption of iron and heme metabolisms in bacteria.

In summary, we evaluated *in vitro* synergistic activity of Ga(III) porphyrin (GaPP or GaMP) in combination with $Ga(NO_3)_3$ against *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, and MRSA in iron-depleted conditions as well as against *in vitro* biofilms formed on plasma-coated surfaces and *in vivo C. elegans* infection models. The Ga(III) porphyrin/$Ga(NO_3)_3$ combination demonstrated substantial synergism against *K. pneumoniae*, *P. aeruginosa*, and MRSA while moderate synergism and indifferent effect were observed against *A. baumannii*. The combination inhibition of Ga-based compounds disrupted both iron-driven redox processes and functions of hemoproteins/enzymes such as cytochrome and catalase and has potential therapeutic index to prevent multi-drug resistant pathogens (Figure 6). The GaPP/$Ga(NO_3)_3$ combination significantly disrupted *K. pneumoniae* and *P. aeruginosa* biofilms and also increased the survival of *C. elegans* infected with *K. pneumoniae* or *P. aeruginosa*. The GaPP/colistin combination also showed synergistic activity against *K. pneumoniae* and *P. aeruginosa*. Future studies are required to assess the effect of combination inhibition in *in vivo* infection models and it is

currently being tested in mice to validate the synergistic activity. Similarly, GaPP has yet to be studied in humans and approved for clinical use.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

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Notes

The authors declare no competing financial interest except that BEB is a co-inventor on patents for gallium therapy held by the University of Iowa Research Foundation.

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