DRUG DEVELOPMENT

Gallium disrupts bacterial iron metabolism and has therapeutic effects in mice and humans with lung infections

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The lack of new antibiotics is among the most critical challenges facing medicine. The problem is particularly acute for Gram-negative bacteria. An unconventional antibiotic strategy is to target bacterial nutrition and metabolism. The metal gallium can disrupt bacterial iron metabolism because it substitutes for iron when taken up by bacteria. We investigated the antibiotic activity of gallium ex vivo, in a mouse model of airway infection, and in a phase 1 clinical trial in individuals with cystic fibrosis (CF) and chronic *Pseudomonas aeruginosa* airway infections. Our results show that micromolar concentrations of gallium inhibited *P. aeruginosa* growth in sputum samples from patients with CF. Ex vivo experiments indicated that gallium inhibited key iron-dependent bacterial enzymes and increased bacterial sensitivity to oxidants. Furthermore, gallium resistance developed slowly, its activity was synergistic with certain antibiotics, and gallium did not diminish the antibacterial activity of host macrophages. Systemic gallium treatment showed antibiotic activity in murine lung infections. In addition, systemic gallium treatment improved lung function in people with CF and chronic *P. aeruginosa* lung infection in a preliminary phase 1 clinical trial. These findings raise the possibility that human infections could be treated by targeting iron metabolism or other nutritional vulnerabilities of bacterial pathogens.

INTRODUCTION

In addition to treating primary infection, antibiotics provide a protective umbrella under which much of modern medicine operates. Patients undergoing surgery, invasive procedures, immune modulation, and cancer therapy all depend upon antibiotics. However, antibiotic effectiveness is threatened. Among the most pressing challenges are escalating antibiotic resistance of both hospital- and community-acquired organisms (*1*, *2*) and the increasing prevalence of pathogens with high intrinsic resistance (*3*). In addition, antibiotics work poorly against chronic infections because the bacterial growth mode at chronic infection sites produces an antibiotic-tolerant phenotype (*4*). The problem is particularly acute for Gram-negative bacteria because of their low cell wall permeability and effective and redundant efflux systems (*5*, *6*).

An unconventional approach to combat infection is to exploit nutritional vulnerabilities of bacteria, and bacterial iron metabolism is a prime candidate. Iron is essential for almost all pathogens because it is required in enzymes mediating DNA synthesis, electron transport, oxidative stress defense, and other key processes (*7*). Moreover, free iron concentrations are extremely low in vivo (~10^{-20} M) because of the insolubility of iron in aerobic environments and the multiple host defenses that sequester iron (*7*). In addition, in vitro work indicates that iron metabolism may be a particular vulnerability for organisms in biofilm-like aggregates found at sites of chronic infections in people with wounds, cystic fibrosis (CF), and other conditions (*8*). Despite these factors, approved therapeutics targeting bacterial iron metabolism have not yet been developed.

One potential approach uses the metal gallium as a "Trojan horse" to disrupt iron metabolism. Gallium has a nearly identical ionic radius as iron, and some bacterial uptake systems are unable to distinguish gallium from iron (*9*, *10*). Gallium disrupts iron-dependent processes because it cannot be reduced in physiological conditions, and iron's biological functions involve redox cycling (*10*). Thus, gallium incorporation into iron-containing proteins disrupts their functioning.

Previous work by others and us found that gallium compounds had antibacterial activity against a number of human pathogens including *Pseudomonas aeruginosa* (*11*), *Francisella tulerensis* (*12*), *Acinetobacter baumannii* (*13*), several mycobacterial species (*14*, *15*), *Klebsiella pneumoniae* (*16*, *17*), and other important pathogens (*18*–*20*). Work with *P. aeruginosa* showed that gallium was effective against bacteria grown as biofilms, in stationary-phase cultures, and against multidrug-resistant CF clinical isolates (*11*).

Here, we tested gallium's effectiveness as an anti-infective treatment. We measured gallium's activity in human CF sputum, performed experiments to understand gallium's mechanism of action, investigated the potential for gallium resistance, and studied gallium's combined activity with conventional antibiotics. We also tested gallium in murine infections and report the results of a

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proof-of-principle human phase 1 trial evaluating gallium in people with CF and chronic *P. aeruginosa* lung infections.

RESULTS

Iron is a growth-limiting nutrient in CF sputum

Previous work shows that high ambient iron concentrations can reduce gallium uptake by *P. aeruginosa* and blunt gallium's antibacterial activity (*11*). These finding suggest that gallium's activity could be enhanced in CF if iron was a growth-limiting nutrient in CF sputum. We tested this in two ways. First, we investigated whether exogenous iron addition would increase *P. aeruginosa* growth in CF sputum. We prepared CF sputum for bacterial growth measurements by mixing freshly expectorated samples 1:1 with saline and then removed solids and endogenous bacteria by centrifugation followed by filtration. Iron addition in the form of iron trichloride ($FeCl_3$) markedly increased both *P. aeruginosa* growth rate and cell yield in sputum obtained from four patients with CF (Fig. 1A and figs. S1 and S2).

Second, we investigated whether bacterial growth in sputum induced bacterial iron starvation genes. We used *P. aeruginosa* expressing a fluorescent reporter linked to the gene that encodes the key *P. aeruginosa* iron uptake protein pyoverdine (strain *pvdA-gfp*), whose production is induced under iron-limited conditions (*21*), and by directly measuring pyoverdine. As shown in Fig. 1 (B and C) and fig. S1 (B and C), *pvdA* and pyoverdine were highly induced during growth in sputum, and expression was repressed by adding exogenous iron. These findings, along with previous work measuring bacterial gene expression (*22*, *23*), indicate that iron is a growth-limiting nutrient for *P. aeruginosa* in CF sputum.

Iron-limiting conditions in sputum enhance gallium's antibacterial effect

To determine how iron concentrations in CF sputum affect gallium's activity, we measured gallium's antibacterial effect in CF sputum with and without iron addition. In the absence of added iron, 4.0 or 5.0 μM gallium completely inhibited *P. aeruginosa* growth in all sputum samples we tested, and in some samples, gallium was effective at 10-fold lower concentrations (Fig. 2A and fig. S3). Adding growth-stimulatory quantities of iron to sputum decreased the activity of the lowest gallium concentrations we tested; however, gallium concentrations that strongly suppressed growth in the unsupplemented condition (≤4.0 μM) were still effective after $FeCl_3$ addition (Fig. 2B).

Gallium inhibits some iron-containing enzymes in *P. aeruginosa*

We studied gallium's mechanism of action against *P. aeruginosa* by investigating gallium's effect on key iron-containing enzymes. The iron-dependent enzyme ribonucleotide reductase is essential for DNA synthesis, and gallium has been shown to inhibit *Mycobacterium tuberculosis* growth by inhibiting cellular ribonucleotide reductase activity (*15*). We investigated gallium's effect in an iron-rich media in which gallium's antimicrobial activity was inhibited to reduce the chance that enzyme activity measurements were confounded by nonspecific changes associated with bacterial death.

As shown in Fig. 3A and consistent with our previous observations with *M. tuberculosis* (*15*), gallium progressively inhibited *P. aeruginosa* ribonucleotide reductase activity reaching a maximum of ~40% inhibition at a gallium concentration of 20 μM in this

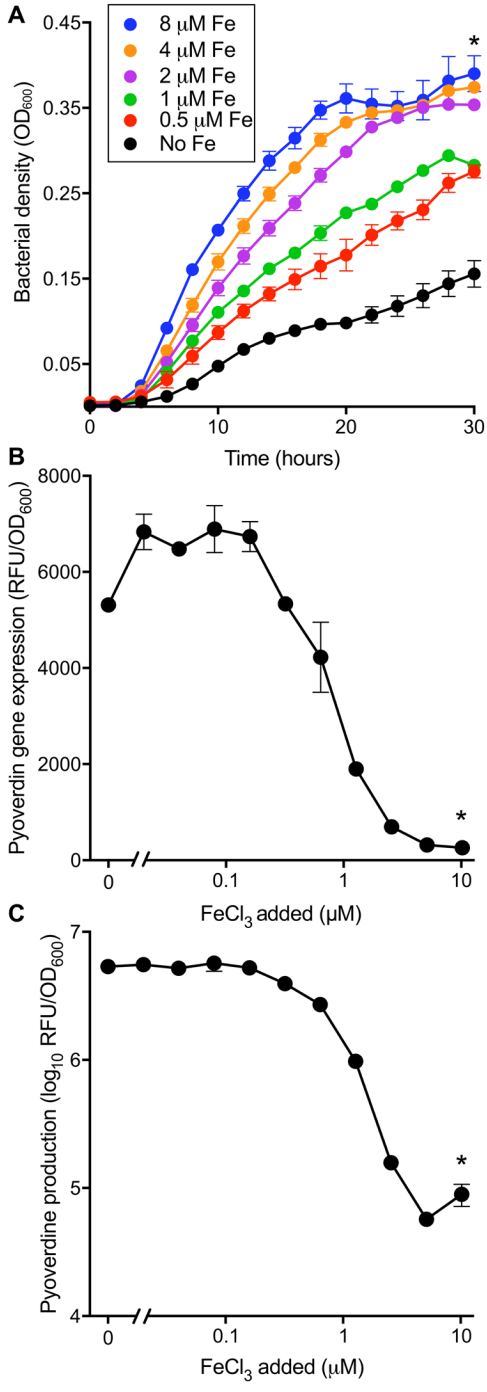

Fig. 1. Expectorated sputum from CF patients is iron-limited. (**A**) Effect of adding iron trichloride ($FeCl_3$) on the growth rate and cell yield of *P. aeruginosa* in sputum supernatants. Results are representative of five sputum samples (see figs. S1 and S2). Error bars indicate SEM. *$P < 0.01$ versus no iron addition, Student's *t* test. (**B** and **C**) Effect of adding iron trichloride ($FeCl_3$) on the expression of the pyoverdine biosynthetic gene *pvdA* (B) and on pyoverdine production (C) by *P. aeruginosa* in CF sputum. Results are mean of two replicates. Error bars indicate SEM. *$P < 0.01$ versus no iron addition, Student's *t* test (also see fig. S1 for experiments with sputum from another subject). OD_{600}, optical density at 600 nm; RFU, relative fluorescence units.

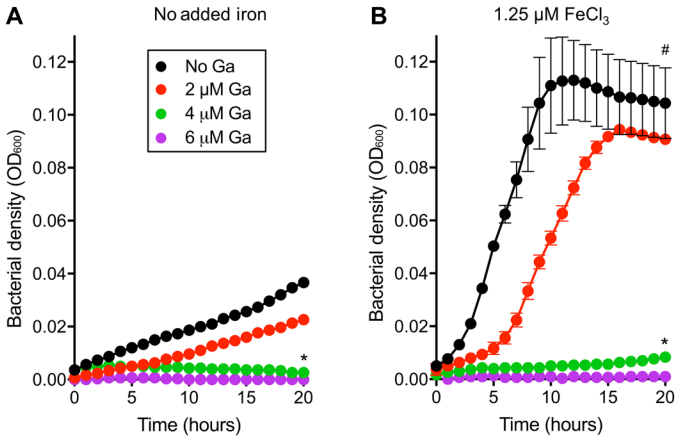

Fig. 2. Gallium inhibits *P. aeruginosa* growth in CF sputum. Gallium's effect on *P. aeruginosa* growth and cell yield in CF sputum supernatants that were not (**A**) and were (**B**) supplemented with iron trichloride ($FeCl_3$). Results are mean of three replicates. *$P < 0.01$ versus no gallium, Student's *t* test; #$P < 0.01$ versus no added iron, Student's *t* test.

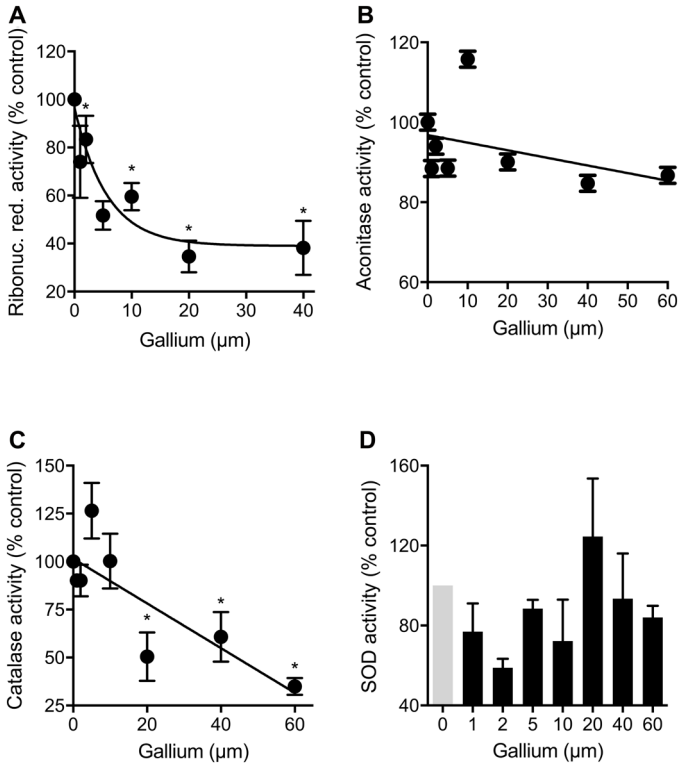

Fig. 3. Gallium inhibits *P. aeruginosa* catalase and ribonucleotide reductase, but not SOD or aconitase activity. Effect of gallium on the activity of ribonucleotide reductase (**A**), aconitase (**B**), catalase (**C**), and SOD (**D**). Results shown are representative of 3 to 37 experiments and are mean enzyme activity measurements relative to bacteria not treated with gallium. Error bars indicate SEM. *$P < 0.05$ versus no gallium, analysis of variance (ANOVA).

medium. However, further inhibition was not seen when the gallium concentration was increased (Fig. 3A). This result raises the possibility that gallium inhibits one of the two classes of *P. aeruginosa* ribonucleotide reductase (*24*), but not the other.

We also exposed live bacteria to gallium to investigate gallium's effect on the activity of aconitase, an iron-sulfur enzyme that catalyzes the isomerization of citrate to isocitrate in the tricarboxylic acid cycle (*15*). Previous work indicated that exposure to gallium decreased *M. tuberculosis* aconitase activity (*15*). However, we found no effect of gallium on *P. aeruginosa* aconitase activity, even after a 24-hour incubation with up to 60 μM gallium (Fig. 3B), which inhibited bacterial growth in this medium.

Catalase and iron–superoxide dismutase (Fe-SOD) are key bacterial antioxidant enzymes that contain iron in their active sites. Consistent with our previous results with *Francisella novicida* (*12*), incubation of *P. aeruginosa* in the presence of increasing concentrations of gallium decreased *P. aeruginosa* catalase activity up to 70% (Fig. 3C). However, in these assay conditions, we found no detectable inhibition of *P. aeruginosa* SOD activity (Fig. 3D).

Gallium increases *P. aeruginosa* oxidant sensitivity

Gallium-mediated inhibition of catalase activity could increase bacterial sensitivity to oxidants, which are key effectors of epithelial and phagocyte-mediated bacterial killing (*25*). To explore this possibility, we exposed *P. aeruginosa* to subinhibitory concentrations of gallium and measured the sensitivity of *P. aeruginosa* to killing by oxidants. Gallium exposure increased *P. aeruginosa* sensitivity to hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide (tert-butyl) (Fig. 4, A and B). In contrast, gallium did not increase sensitivity to paraquat (PQ) or phenazine methosulfate (PMS) (Fig. 4, C and D), which primarily generate superoxide. These findings are consistent with our data indicating that gallium inhibits *P. aeruginosa* catalase that catabolizes H_2O_2, but not SOD that converts superoxide to H_2O_2.

P. aeruginosa develops gallium resistance at rates comparable to successful antibiotics

Most successful antibiotics inhibit multiple essential bacterial functions, and this may slow drug resistance (*26*). For example, ciprofloxacin targets DNA gyrase and topoisomerase, and β-lactam antibiotics target multiple penicillin-binding proteins (*26*). The fact that gallium can substitute for iron in many proteins and interferes with multiple bacterial functions (see Figs. 3 and 4) led us to hypothesize that *P. aeruginosa* may develop resistance to gallium at low rates, similar to successful antibiotics that have multiple targets.

We compared the frequency at which *P. aeruginosa* develops spontaneous resistance to gallium and to the conventional antipseudomonal antibiotics colistin, ciprofloxacin, and tobramycin. Spontaneous resistance was defined as the heritable ability to grow in the presence of four times the minimal inhibitory concentration (MIC) of each agent. About 1 in 30 million *P. aeruginosa* cells spontaneously developed resistance to gallium (Table 1). Spontaneous resistance to the other antibiotics tested occurred about two times more frequently (Table 1).

We also measured the rate at which mutations arise under selection by passaging 12 replicate cultures of wild-type *P. aeruginosa* in gallium, aztreonam, and tobramycin for 12 days. Whereas resistance to gallium and the tested antibiotics increased significantly after passaging ($P < 0.01$), relative gallium resistance increased less ($P < 0.01$). (Fig. 5, A to C). The relatively low rates of gallium resistance in both assays are consistent with our previous finding that except for one outlier (of 115 strains tested), the most resistant *P. aeruginosa* clinical isolate we tested had an inhibitory concentration only fourfold higher than the gallium-susceptible laboratory strain, PA01 (*11*).

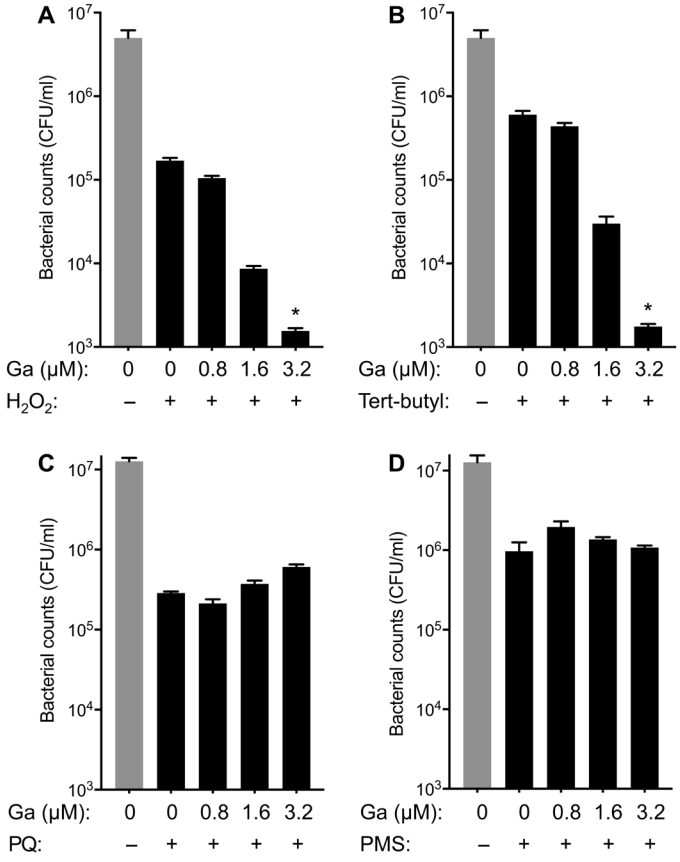

Fig. 4. Gallium increases *P. aeruginosa* sensitivity to peroxides. Effect of subinhibitory concentrations of gallium on *P. aeruginosa*'s sensitivity to oxidants generating peroxide, including H_2O_2 (**A**) and tert-butyl hydroperoxide (tert-butyl) (**B**); and superoxide, including PQ (**C**) and PMS (**D**). Subinhibitory gallium increased sensitivity to killing by peroxides. Data are mean values of three to four replicate experiments. Error bars indicate SEM. *$P < 0.01$, Student's *t* test. CFU, colony-forming units.

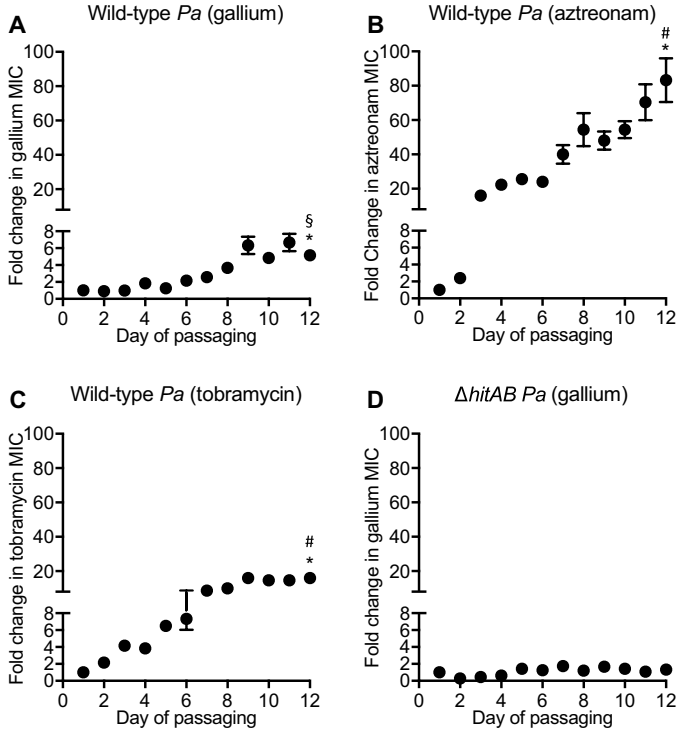

Fig. 5. Continuous passaging increases gallium and antibiotic resistance. Effect of passaging wild-type (**A** to **C**) and Δ*hitAB P. aeruginosa* (**D**) in gallium (A and D), aztreonam (B), and tobramycin (C). The mean fold change in highest drug concentration that permitted growth (of 12 replicate cultures) is plotted as a function of the passaging day. Error bars indicate SEM. *$P < 0.01$ versus the highest drug concentrations that permitted growth before passaging (see Methods), Wilcoxon matched-pairs signed-rank test. #$P < 0.01$ versus the fold change of wild-type *P. aeruginosa* after passaging in gallium, Mann-Whitney. §$P < 0.01$ versus the fold change of Δ*hitAB P. aeruginosa* after passaging in gallium, Mann-Whitney.

Table 1. Frequency of spontaneous *P. aeruginosa* mutants.

Antibacterial agent	Mutation frequency
Gallium (64 μM)	$2.90 \times 10^{-8} \pm 1.35 \times 10^{-8}$
Tobramycin (4 μg/ml)	$3.52 \times 10^{-8} \pm 1.80 \times 10^{-8}$
Colistin (16 μg/ml)	$5.80 \times 10^{-8} \pm 3.00 \times 10^{-9}$
Ciprofloxacin (4 μg/ml)	$8.48 \times 10^{-8} \pm 2.13 \times 10^{-8}$

Transposon mutagenesis identifies few gene inactivation producing gallium resistance

Recent work using transposon mutagenesis in *P. aeruginosa* PA14 found that inactivation of the *hitA* gene, which encodes a periplasmic $iron^{+3}$ transporter, produced fourfold reductions in gallium sensitivity (*27*). We used three approaches to determine whether additional resistance-producing gene inactivation mutations could be identified.

First, because resistance elements may be strain-specific, we repeated transposon mutagenesis using the *P. aeruginosa* strain PA01, which is the reference strain most phylogenetically related to CF clinical isolates and is among the most divergent reference strain from PA14 (*28*, *29*). Genome saturation-scale transposon mutagenesis in PA01 (total of ~120,000 mutants screened) found no additional mutants [other than *hitA*, as found by (*27*)] to be associated with gallium resistance (table S1).

Second, we performed genome-saturating transposon mutagenesis in a PAO1 strain in which the *hitAB* genes had been deleted. This screen of ~240,000 transposon mutants found only two mutants with gallium resistance higher than the *hitAB* deletion strain (table S1). The transposon insertions were mapped to the open reading frame of *PA5248*, which has homology to an inner membrane iron permease gene (the FTR1/Fip1/EfeU family), and to the intergenic region between the *pvdA* and *fpvI* genes, both of which are involved in iron acquisition (*21*). Adding transposon mutations in *PA5248* or the *pvdA-fpvI* intergenic region to the *hitAB* deletion strain increased resistance by only about twofold (fig. S4).

Third, we passaged 12 replicate cultures of the *P. aeruginosa hitAB* deletion strain in gallium for 12 days and found that whereas this strain exhibited a higher starting inhibitory concentration than wild type, prolonged passaging produced smaller relative increases in gallium resistance as compared to wild type ($P < 0.0001$) (Fig. 5D). Together, this work suggests that marked gallium resistance is not likely to occur at high frequencies and that inactivation of the *hitAB* iron transporter is the main pathway to resistance.

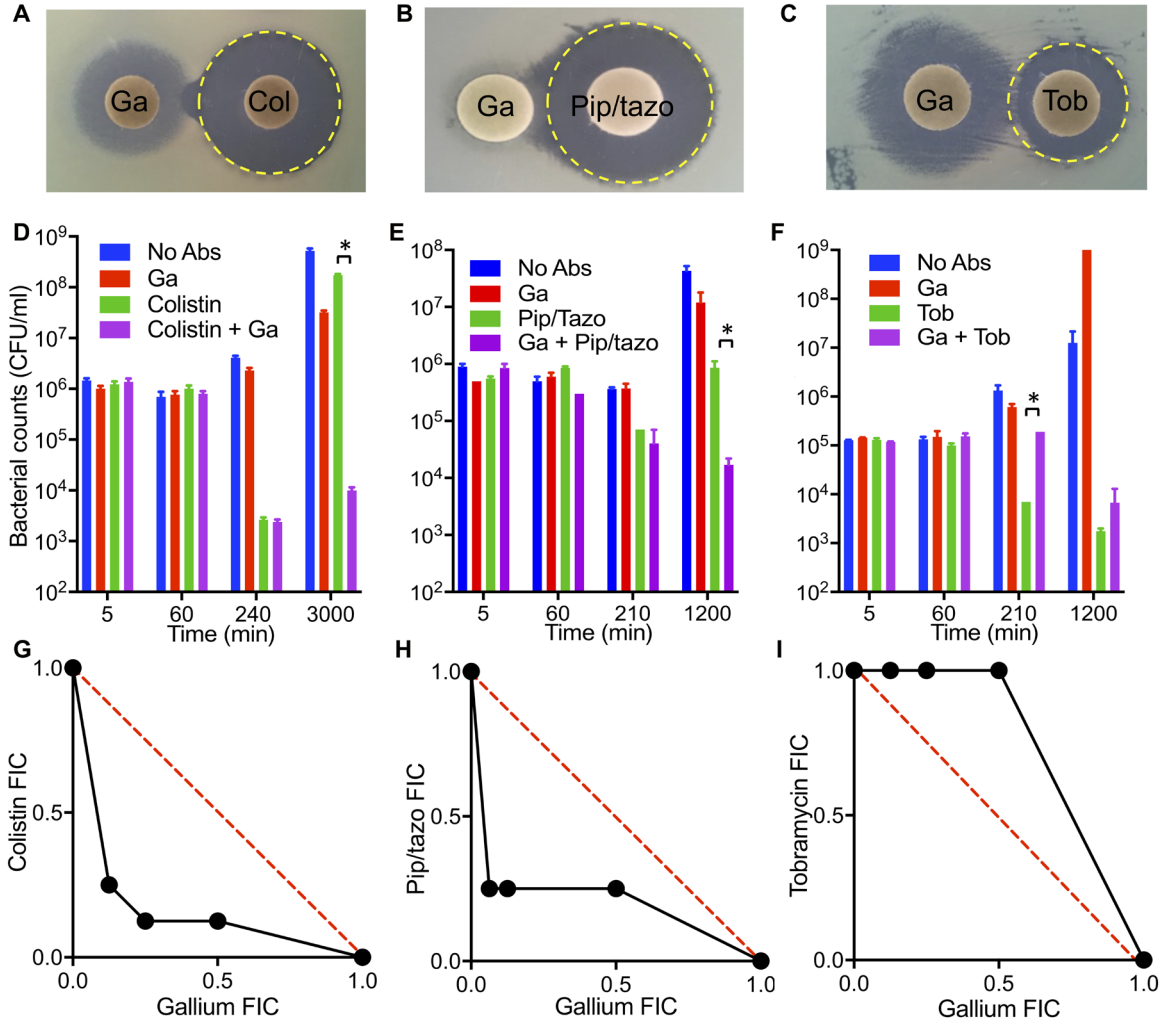

Fig. 6. Gallium has synergistic activity with antibiotics. Combined effect of gallium with colistin (**A**, **D**, and **G**), piperacillin/tazobactam (**B**, **E**, and **H**), and tobramycin (**C**, **F**, and **I**). Photographs (A to C) show disc diffusion assays. The yellow dashed lines represented the expected activity (in preventing *P. aeruginosa* growth) of the antibiotic in the absence of gallium. Graphs (D to F) show time-kill assays using subinhibitory concentrations of gallium and inhibitory concentrations of antibiotics. Error bars indicate SEM. *$P < 0.05$, Student's *t* test. Isobolograms (G to I) show results of checkerboard assays presented as the fractional inhibitory concentrations (FICs) of the two factors in combination. Calculations are described in Methods. Experiments were repeated two to four times, each with similar results. Abs, antibiotics.

Gallium is synergistic with two anti-pseudomonal antibiotics

Gallium's unique mechanism makes its combined activity with antibiotics difficult to predict. We used three independent assays to measure the combined activity of gallium and antibiotics including the agar disc diffusion, time-kill, and checkerboard (isobologram) assays (*30*).

All three assays detected synergistic interactions between gallium and colistin (polymyxin E) and gallium and piperacillin/tazobactam. Synergy was indicated by convex inhibition zones between gallium and antibiotic discs in disc diffusion assays (Fig. 6, A and B), increased bactericidal activity in time-kill assays (Fig. 6, D and E), and concave-shaped isobolograms in checkerboard assays (Fig. 6, G and H). In contrast, gallium was antagonistic to tobramycin's activity (Fig. 6 C, F, and I), and neither synergistic nor antagonistic interaction effects were seen with ciprofloxacin, aztreonam, or ceftazidime (fig. S5). These findings could inform future clinical studies that combined gallium with conventional antibiotics.

Gallium does not inhibit the antimicrobial activity of macrophages

Gallium is used clinically to treat hypercalcemia of malignancy because it inhibits bone reabsorption by osteoclasts (*10*, *29*), which are myeloid cells. Macrophages (also myeloid cells) are present in chronically infected CF airways and take up gallium (*31*). These facts raise concern that gallium could negatively affect macrophage function. To test this, we isolated human monocytes, differentiated them to human monocyte-derived macrophages (HMDMs), and treated the HMDMs with vehicle or gallium. We used a long exposure (24 hours) and a gallium (100 μM) concentration that was ~20 to 200 times the concentration that inhibited *P. aeruginosa* in sputum and ~10 times the concentration detected in sputum in the clinical trial (see below) for these experiments to maximize the chances of detecting toxic effects.

Gallium treatment did not reduce HMDM viability (fig. S6A), but it modestly affected the expression of some genes mediating bacterial uptake and killing (fig. S6B). We directly tested gallium's

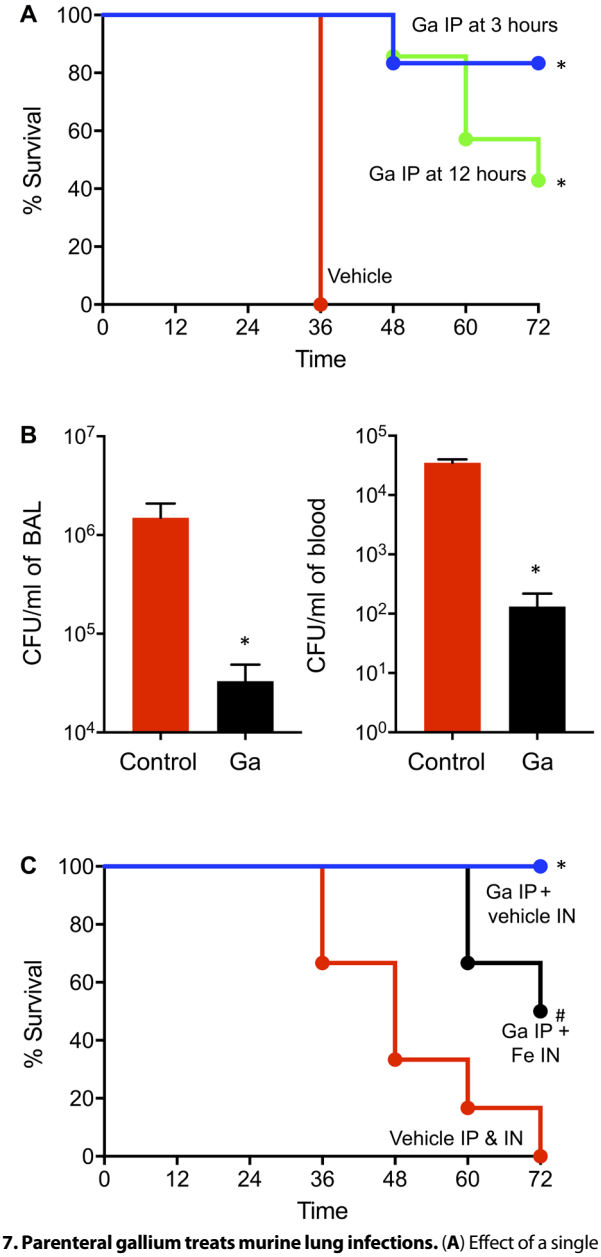

Fig. 7. Parenteral gallium treats murine lung infections. (**A**) Effect of a single intraperitoneal (IP) dose of gallium-free vehicle (red line) or gallium [50 μl of 250 mM $Ga(NO_3)_3$] administered 3 hours (blue line) or 12 hours after (green line) intratracheal infection with *P. aeruginosa* ($n = 7$ mice for gallium and $n = 8$ mice for vehicle). *$P <$ 0.001 versus vehicle control, Fisher test. (**B**) *P. aeruginosa* counts in bronchoalveolar lavage (BAL) fluid and blood 12 hours after mice were infected by the intratracheal route and treated with vehicle (intraperitoneal) or gallium (intraperitoneal) 3 hours after infection ($n = 4$ mice for vehicle alone and $n = 5$ mice for gallium). *$P < 0.001$ versus vehicle control, Fisher test. (**C**) Effect of intranasal (IN) iron-free vehicle (blue line) or iron (10 μl of 2 mM $FeCl_3$) (black line) on the antibiotic effect of intraperitoneal gallium. *$P < 0.001$ versus vehicle control, Fisher test; #$P < 0.05$ versus vehicle control, Fisher test. The red line shows mouse survival without gallium (vehicle administered intraperitoneally and intranasally) ($n = 6$ mice in each group).

effects on macrophage antimicrobial activity using HMDMs isolated from two healthy donors and found that continuous exposure to 100 μM gallium for 24 hours did not reduce macrophage *P. aeruginosa* killing (fig. S6C).

Parenteral gallium effectively treats *P. aeruginosa* mouse lung infections

We focused on proof-of-principle in vivo studies (in mice and humans) on systemic rather than inhaled gallium treatment because intravenous gallium nitrate [$Ga(NO_3)_3$] is already approved by the U.S. Food and Drug Administration for a noninfection indication (hypercalcemia of malignancy). We began by testing parenteral gallium in a mouse model of *P. aeruginosa* lung infections using a single dose administered 3 or 12 hours after mice were infected with *P. aeruginosa*. Gallium treatment increased mouse survival ($P <$ 0.001) (Fig. 7A) and reduced lung and blood *P. aeruginosa* counts ($P < 0.001$) (Fig. 7B).

To determine whether disrupted iron metabolism explained gallium's efficacy, we exogenously added an iron solution into mouse airways immediately before infection and found that iron addition reduced gallium's therapeutic effect ($P < 0.001$) (Fig. 7C). These data show that systemic gallium is effective in a model acute lung infection (even when administered well after the bacteria) and suggest that as seen in vitro, gallium's in vivo activity results from disruption of iron-dependent processes.

A proof-of-principle phase 1 clinical trial of gallium in CF

Given in vitro and in vivo preclinical data suggesting efficacy, we tested intravenous gallium administration in people with CF and chronic *P. aeruginosa* lung infections in a pilot phase 1b nonrandomized study (investigator-initiated: IND #104,363; ClinicalTrials.gov: NCT01093521). We enrolled 20 patients, evenly divided between genders, with a median age of 32.8 years (range, 19 to 54.2) and a mean forced expiratory volume in 1 s (FEV_1) of 2.24 liters (range, 1.06 to 4.59 liters) (see tables S2 and S3 for inclusion and exclusion criteria and enrollment).

The primary endpoints were safety, tolerability, and pharmacokinetics; however, we also measured efficacy endpoints including change in lung function as measured by FEV_1 and forced vital capacity (FVC) (from baseline to days 7, 14, and 28) and change in sputum *P. aeruginosa* density (from baseline to days 7, 14, and 28). We sequentially studied two dose regimes to enable early detection of adverse effects. Cohort 1 received a low dose, 100 mg/m^2 per day (9 subjects: 4 males and 5 females), and cohort 2 received 200 mg/m^2 per day (11 subjects: 6 males and 5 females).

No serious adverse events were noted, and kidney function, electrolyte concentrations, and blood counts were all unaffected (table S4 and fig. S7). Steady-state plasma and sputum concentrations were achieved by 2 days (Fig. 8, A and B, and fig. S8). Gallium plasma and sputum area under the curve (AUC) concentrations did not change with increased dose (table S5), likely because gallium's saturable protein binding [for example, to transferrin and albumin (*32*)] causes unbound gallium concentrations to increase at higher doses, which, in turn, increases renal and nonrenal elimination (*33*).

Plasma and sputum gallium concentrations remained detectable for prolonged periods (Fig. 8, A and B, and fig. S8). The average plasma and sputum elimination half-life ($T_{1/2\beta}$) of gallium exceeded 100 and 220 hours, respectively, in both cohorts (table S5). Sputum concentration increased after the end of the infusion, increasing, on average, more than twofold by day 14, and only decreased by ~50% on day 28 (Fig. 8B and table S5). These findings suggest that a depot compartment exists, which could explain the prolonged improvement in lung function we observed (see below).

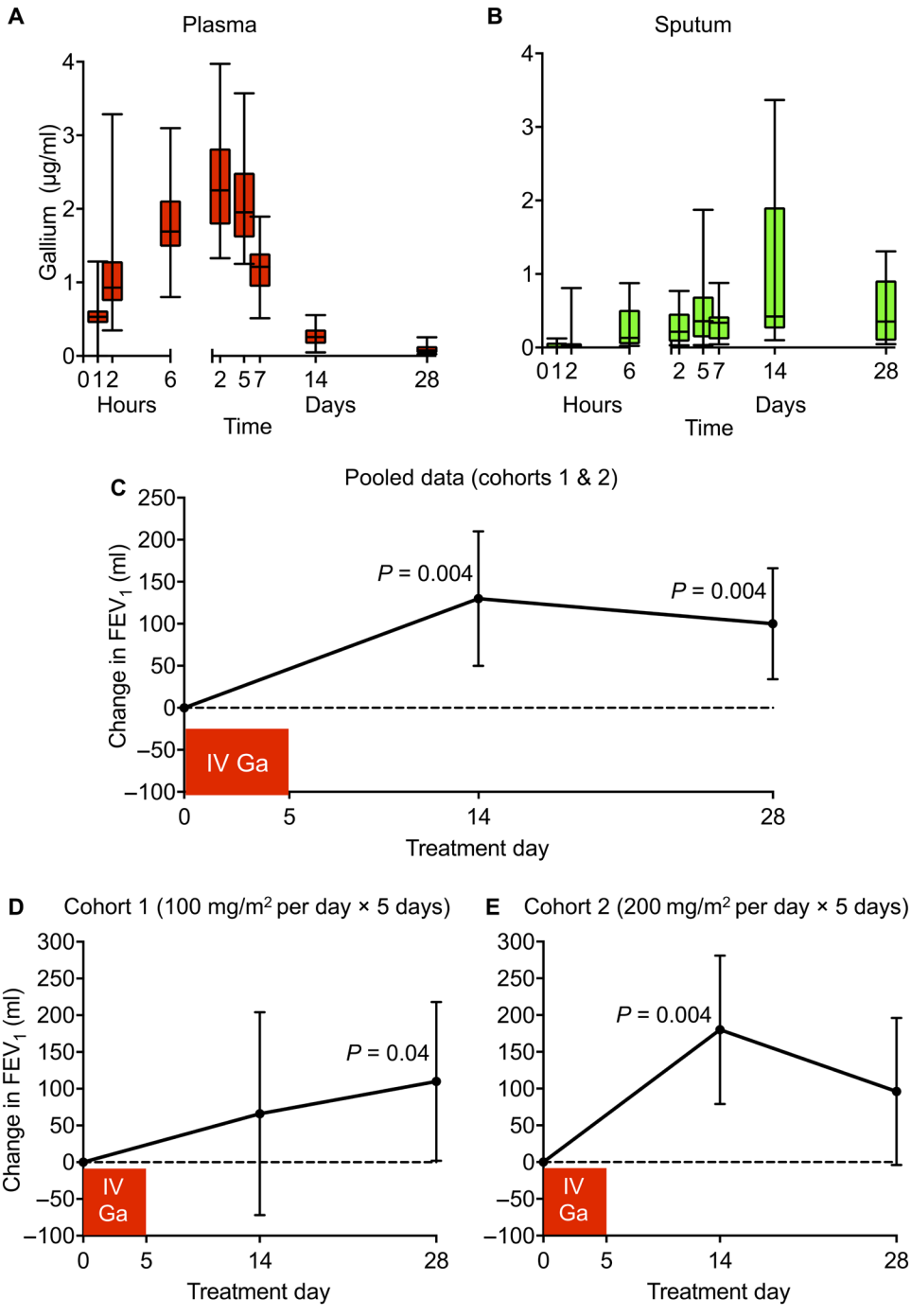

Fig. 8. Intravenous gallium produces sustained blood and sputum concentrations and improves lung function. Plasma (**A**) and sputum (**B**) gallium concentrations in CF subjects treated with intravenous gallium for 5 days; boxes show 25th to 75th percentiles, hatches shows means, and whiskers show minimum and maximum values. Data shown are for cohorts 1 and 2 (100 mg/m^2 per day and 200 mg/m^2 per day, respectively) combined (see fig. S8 for data from each cohort separated). Mean change in lung function (as measured by FEV_1 in mls) by study day for both cohorts pooled together (**C**) and cohorts 1 (**D**) and 2 (**E**) separately (100 mg/m^2 per day and 200 mg/m^2 per day, respectively). Bars represent the 95% confidence intervals (CIs). *P* values are indicated on graphs. Inferential analyses of the change from baseline tested the null hypothesis that the change is equal to zero using paired *t* tests with normally distributed data with 95% CIs.

Analysis of pooled data (from cohorts 1 and 2) showed statistically significant increases in lung function (both FEV_1 and FVC) 14 and 28 days after a single infusion of gallium ($P < 0.005$) (Fig. 8C, Table 2, and table S6). Data from the individual cohorts are shown in Fig. 8 (D and E) and table S6. When defined by the proportion of subjects achieving a 5% FEV_1 improvement, 73 and 45% of cohort 2 were responders at days 14 and 28, respectively, compared to 22 and 44% of cohort 1 at days 14 and 28, respectively (*P* values in table S6). However, we found no correlation between lung function response and peak or AUC sputum gallium concentrations (table S7). Mean and median *P. aeruginosa* density declined by 5.5 million and 1.8 million CFU/g between days 0 and 14 and by 29.8 million and 3.9 million CFU/g between days 0 and 28 (Table 2), but these changes were not statistically significant.

DISCUSSION

Whereas the idea of disrupting bacterial nutrition as an antimicrobial strategy was raised by Louis Pasteur in the 1800s (*34*), therapeutic approaches that exploit bacterial nutrient vulnerabilities have been difficult to develop. Our results indicate that gallium can act as a chemical mimic to disrupt *P. aeruginosa* iron metabolism, that gallium resistance develops at low rates, and that gallium's antibacterial activity may be enhanced by some conventional antibiotics. Furthermore, our preliminary proof-of-concept clinical study raises the possibility that gallium may be a safe and effective treatment for human infections.

Gallium's ability to generally substitute for iron suggests that gallium could disrupt several aspects of bacterial physiology, and our experiments and previous gene expression analysis (*11*) support this idea. Activity assays indicated that gallium inhibits enzymes mediating bacterial DNA synthesis and antioxidant defense and sensitized bacteria to killing by peroxides. Previous gene expression assays indicated that gallium disrupts carbon utilization and protein synthesis and represses key iron uptake systems including those mediating heme/hemoglobin and pyoverdine uptake (*21*). The finding that gallium suppresses iron uptake and our data indicating that

Table 2. Lung function and microbiology in CF patients at baseline, day 14, and day 28. NA, not applicable; IQR, interquartile range.

Endpoint	Mean and median baseline (day 0)	Mean and median change from days 0–14	*P*	Mean and median change from days 0–28	*P*
All patients (*n* = 20)					
FEV_1 (liter)					
Mean (SD)	2.24 (0.84)	0.13 (0.17)	0.0041	0.10 (0.14)	0.0041
Median (IQR)	2.1 (1.68 to 2.58)	0.075 (0.015 to 0.22)	0.0064	0.085 (0.00 to 0.18)	0.0042
Proportion with 5% change in FEV_1 (95% CI)	NA	50% (27 to 73%)		45% (23 to 69%)	
FVC (liter)					
Mean (SD)	3.58 (1.0)	0.13 (0.15)	0.0010	0.16 (0.15)	0.0001
Median (IQR)	3.53 (3.14 to 4.04)	0.12 (0.01 to 0.23)	0.0007	0.15 (0.045 to 0.24)	0.0001
Sputum *P. aeruginosa* (million CFU/gm)	*n* = 19	*n* = 19	0.5949	*n* = 17	0.5171
Mean (SD)	117 (200)	−5.51 (218)	0.9134	−29.8 (171)	0.4825
Median (IQR)	48.4 (5.5 to 137.5)	−1.83 (−87 to 74)		−3.9 (−105 to 55)	

low iron concentrations enhances gallium's activity raises the possibility that gallium effects on bacterial physiological might potentiate its own activity.

The multiplicity of gallium effects could provide advantages of target redundancy, which could explain our finding that gallium resistance emerged at low rates, comparable to antibiotics that target multiple processes. Also promising, the bacterial functions that we identified as being gallium-inhibited are not targeted by antibiotics in clinical use. This could explain our previous finding that multidrug-resistant *P. aeruginosa* isolates showed similar degree of gallium sensitivity as antibiotic-sensitive laboratory strains (*11*).

The magnitude of gallium-associated lung function improvement we identified in the pilot clinical trial is similar to that produced by approved antibiotics in CF. For example, 14-day-long treatment using a combination of two intravenous anti-pseudomonal antibiotics chosen on the basis of the antimicrobial susceptibility of subject's *P. aeruginosa* strains improved FEV_1 by ~7%, when measured immediately (12 to 48 hours) after the last antibiotic dose (*35*). Likewise, high doses of tobramycin and aztreonam administered by inhalation twice or three times daily for month-long courses improved FEV_1 by 2.7 to 10.3%. The lung function improvements produced by conventional antibiotics in these studies waned 2 to 4 weeks after the drug was stopped (*36*–*39*), whereas gallium-associated changes persisted to the last time point we measured (day 28).

Although the results were encouraging, our study had several limitations. First, the sputum activity, mechanism of action, resistance and synergy, and animal experiments were performed with the *P. aeruginosa* strain, PA01, and it is possible that different strains could give different results. This limitation is mitigated somewhat because PA01 is the reference strain most phylogenetically related to CF isolates (*28*). Furthermore, our previous work testing 120 CF clinical *P. aeruginosa* isolates (including multidrug-resistant strains) (*11*) found these generally gallium-susceptible. In addition, the results of our phase 1 human trial suggest that gallium activity is not dependent on the presence of a particular *P. aeruginosa* strain because CF subjects are generally infected by different environmental strains.

Second, although we measured resistance using three independent assays (that is, spontaneous mutants, passaging under selection, and transposon mutagenesis), combined action with antibiotics using three independent assays (disc diffusion, time-kill, and checkerboard), and found concordant results, it is possible that these do not reflect in vivo activity. Third, although gallium treatment produced statistically significant improvements in lung function (despite the small numbers of subjects studied), declines in *P. aeruginosa* burden did not reach significance. The absence of statistically significant reductions in *P. aeruginosa* counts could be due to the variability of enumerating bacterial counts in sputum, or if gallium's beneficial effect on lung function was due to its previously described anti-biofilm (*11*) or anti-inflammatory (*40*–*43*) effects or activity against other bacteria that may coinhabit CF airways.

Fourth, the clinical study was limited to subjects with mild disease, and gallium's efficacy could be reduced in severe CF because lung function and sputum total iron and $iron^{+2}$ concentrations may be inversely correlated (*44*). However, gallium's efficacy was maintained even when growth-stimulating concentrations of iron are added to CF sputum, higher molar ratios of iron (than gallium) are required to restore growth of gallium-treated bacteria (*11*), and gallium inhibits *P. aeruginosa* iron uptake (*11*). It is unclear whether these factors could mitigate the effects of increased in vivo iron availability or increase gallium's efficacy.

Finally, the clinical trial was small, unblinded, and did not have a placebo control. Thus, the promising safety and efficacy results need to be confirmed in a larger placebo-controlled study. It will also be important to study the safety of repeated administration, investigate combined use with antibiotics in humans, and determine whether oral or inhaled treatment is feasible.

The public health crisis of antibiotic resistance has spurred studies of nonconventional antimicrobial approaches. Our proof-of-principle work with *P. aeruginosa* and the fact that gallium has broad-spectrum activity against many extracellular and intracellular pathogens raise the possibility that gallium or other iron-disrupting strategies may be useful in infections caused by a range of resistant organisms. This work also suggests a renewed focus on targeting bacterial nutrition and metabolism to treat infectious diseases.

METHODS

Study design

The objectives of this work were to test gallium as a potential antibacterial treatment for people with CF and chronic *P. aeruginosa* lung infections. We used in vitro experiments to investigate $Ga(NO_3)_3$ mechanism of action, gallium resistance, the combined activity of gallium with antibiotics, and gallium's effect on macrophage activity. We also tested gallium in mice infected with *P. aeruginosa* and in people with CF and chronic *P. aeruginosa* lung infections in a non-randomized phase 1 clinical trial (see below).

Ex vivo assays

Sputum was collected from CF patients and used for growth studies (*45*) using strains PAO1 and PAO1-*pvdA-gfp* (*46*). Basal medium 2 (BM2) medium was used for oxidant sensitivity, time-kill, checkerboard, spontaneous and selected mutation rate assays, and selection of transposon mutants. The highest drug concentrations that permitted growth before passaging (in the selected resistance assays) were 1 μg/ml for tobramycin and aztreonam, 64 μg/ml gallium for wild-type *P. aeruginosa*, and 128 μg/ml gallium for Δ*hitAB P. aeruginosa*. LB agar plates were used in disc diffusion assays. Enzyme activity assays were preformed in 10% tryptic soy broth medium. Transposon mutagenesis was performed using the mini-Tn5-pro delivered on pUT from *Escherichia coli* S17-1 (see the Supplementary Materials for additional information).

Mouse infections

All experiments were approved in advance by the University of Washington Animal Care and Use Committee and used 8- to 12-week-old C57Bl/6 pathogen-free mice (The Jackson Laboratory). Animals were euthanized if they became moribund, distressed, or unable to eat or drink (see the Supplementary Materials for additional information).

Human studies

Macrophage studies were approved by the University of Chicago Institutional Biosafety Committee, and the trial of intravenous $Ga(NO_3)_3$ (ClinicalTrials.gov: NCT01093521) was performed under IND #104,363, with Institutional Review Board (IRB) approval from all three study sites (UW IRB 35876, UI IRB-01 201002774, and JHU IRB5 NA_00044996).

Subjects were included in the phase 1b clinical trial if they were between 18 and 55 years of age, had a confirmed diagnosis of CF with chronic *P. aeruginosa* lung infection, and did not have severe lung dysfunction (FEV_1 > 30% of predicted). In addition, subjects were required to be without clinically significant renal or liver disease and not be experiencing acute disease flares (see tables S3 and S4 for inclusion and exclusion criteria and enrollment).

Gallium can cause nephrotoxicity after large intravenous boluses because rapid infusion saturates the binding capacity of gallium-binding proteins (*10*, *33*). CF patients frequently receive nephrotoxic drugs, so we administered $Ga(NO_3)_3$ by slow continuous intravenous infusion over 5 days, which has been shown to markedly reduce nephrotoxicity as compared to bolus infusion (*10*). Subjects were also instructed to consume 2 liters of fluid above their normal intake during the infusion period. Subjects received a single 5-day infusion of $Ga(NO_3)_3$ (two dosing cohorts noted above). Cohort 2 did not commence until review of cohort 1 safety data by the Data Safety Monitoring Committee.

Statistical analysis

For the laboratory-based studies, we present descriptive statistics including SD and SE. For between-group comparisons, we used Student's *t* test. For non-normally distributed variables, Mann-Whitney *U* test was performed. For the clinical trial, inferential analyses of the lung function changes tested the null hypothesis that the change was equal to zero using paired *t* tests with normally distributed data with 95% CIs and Wilcoxon signed-rank tests with non-normally distributed data with IQR.

Table 2 notes both mean and median values with SD and IQR. Lung function is measured by both FEV_1 and FVC in liters. Quantitative sputum *P. aeruginosa* concentrations are measured in million CFU/gm. We report the proportion of patients with a 5% improvement in FEV_1 because this is considered clinically significant in CF (*36*–*39*). The change in proportion is noted by the 95% CIs of that change. Inferential analyses of the change from baseline tested the null hypothesis that the change is equal to zero using paired *t* tests with normally distributed data with 95% CIs. Wilcoxon signed-rank tests were used with non-normally distributed data with IQR.

SUPPLEMENTARY MATERIALS

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Methods

Fig. S1. Expectorated sputum from CF patients is iron limited.

Fig. S2. Iron increases bacterial growth rate in CF sputum.

Fig. S3. Gallium inhibits *P. aeruginosa* growth in CF sputum.

Fig. S4. Transposon mutations in *PA5248* or the *pvdA-fpvI* intergenic region modestly increases gallium resistance.

Fig. S5. Gallium's activity used in combination with antibiotics.

Fig. S6. Gallium does not attenuate *P. aeruginosa* killing by human macrophages.

Fig. S7. Intravenous gallium produces minimal changes in red blood cell parameters.

Fig. S8. Intravenous gallium produces sustained blood and sputum concentrations.

Table S1. Results of transposon mutagenesis screening for gallium resistant mutants.

Table S2. Full inclusion and exclusion criteria for clinical trial.

Table S3. Enrollment table.

Table S4. Safety laboratory assessments in CF patients at baseline, day 6, day 14, and day 28.

Table S5. Pharmacokinetics of gallium.

Table S6. Lung function and microbiology in CF patients at baseline, day 14, and day 28 by dosing cohort.

Table S7. Correlation of change in lung function with sputum gallium pharmacokinetics.

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H.A.S., Y.K., and R.J.S. designed the in vitro experiments. Y.K., L.K., S.R., K.M., and R.J.S. performed experiments to investigate gallium's activity in sputum, gallium resistance, and the combined effect of gallium with antibiotics. O.O. performed mechanism of action studies. Y.K. performed animal experiments. G.Z., D.M.C., H.A.S., and L.B. performed macrophage studies. C.H.G., M.L.A., D.B.H., M.T., N.L., E.W., G.D.A., and P.K.S. designed the clinical trial. E.C. provided statistical analysis. G.D.A. analyzed pharmacokinetic data. P.K.S., L.B., B.E.B., G.D.A., and C.H.G. wrote the paper. P.K.S. and C.H.G. provided funding. **Competing interests:** P.S. and B.B. are co-inventors on U.S. patent (U.S. patent no. 9,539,367; Gallium Inhibits Biofilm Formation) held by the University of Iowa. **Data and materials availability:** All the data are in the main manuscript or in the Supplementary Materials.

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Gallium disrupts bacterial iron metabolism and has therapeutic effects in mice and humans with lung infections

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Deceiving bacteria with gallium

Bacterial resistance to available antibiotics is emerging worldwide, and there are few new antibiotics in the pipeline. Goss *et al*. have developed an unconventional strategy for treating bacterial infections. They report that disruption of bacterial iron metabolism by substituting iron with the metal gallium resulted in reduced survival of bacteria in vitro. Gallium also showed antibiotic activity against bacteria in sputum samples from patients with cystic fibrosis and in mouse models of airway infection. In a phase 1 clinical trial, gallium had therapeutic effects without toxicity in cystic fibrosis patients infected with *Pseudomonas*, suggesting that gallium may be useful for treating bacterial infections.

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