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Gallium Compounds Exhibit Potential as New Therapeutic Agents against *Mycobacterium abscessus*

Maher Y. Abdalla,[a,c] Barbara L. Switzer,[a,b] Christopher H. Goss,[d,e] Moira L. Aitken,[d] Pradeep K. Singh,[d,f] Bradley E. Britigan[a,b,c]

Research Service, VAMC-Nebraska/Western Iowa, Omaha, Nebraska, USA[a]; Departments of Internal Medicine[b] and Pathology and Microbiology,[c] University of Nebraska Medical Center College of Medicine, Omaha, Nebraska, USA; Departments of Medicine,[d] Pediatrics,[e] and Microbiology,[f] University of Washington, Seattle, Washington, USA

The rapidly growing nontuberculous mycobacterial species *Mycobacterium abscessus* has recently emerged as an important pathogen in patients with cystic fibrosis (CF). Treatment options are limited because of the organism's innate resistance to standard antituberculous antibiotics, as well as other currently available antibiotics. New antibiotic approaches to the treatment of *M. abscessus* are urgently needed. The goal of the present study was to assess the growth-inhibitory activity of different Ga compounds against an American Type Culture Collection (ATCC) strain and clinical isolates of *M. abscessus* obtained from CF and other patients. In our results, using $Ga(NO_3)_3$ and all of the other Ga compounds tested inhibited the growth of ATCC 19977 and clinical isolates of *M. abscessus*. Inhibition was mediated by disrupting iron uptake, as the addition of exogenous iron (Fe) restored basal growth. There were modest differences in inhibition among the isolates for the same Ga chelates, and for most Ga chelates there was only a slight difference in potency from $Ga(NO_3)_3$. In contrast, Ga-protoporphyrin completely and significantly inhibited the ATCC strain and clinical isolates of *M. abscessus* at much lower concentrations than $Ga(NO_3)_3$. In *in vitro* broth culture, Ga-protoporphyrin was more potent than $Ga(NO_3)_3$. When *M. abscessus* growth inside the human macrophage THP-1 cell line was assessed, Ga-protoporphyrin was >20 times more active than $Ga(NO_3)_3$. The present work suggests that Ga exhibits potent growth-inhibitory capacity against the ATCC strain, as well as against antibiotic-resistant clinical isolates of *M. abscessus*, including the highly antibiotic-resistant strain MC2638. Ga-based therapy offers the potential for further development as a novel therapy against *M. abscessus*.

Individuals with cystic fibrosis (CF) suffer from chronic and recurrent bacterial infections of the lung that are, in turn, associated with progressive deterioration of respiratory function (1). *Staphylococcus aureus* is the most commonly isolated pathogen in young children (2), with the proportion of patients colonized with *Pseudomonas aeruginosa* increasing with age (1). Among the organisms of growing importance in recent years is the rapidly growing nontuberculous mycobacterial (NTM) species *Mycobacterium abscessus*.

M. abscessus infects macrophages of the lungs and skin and causes a variety of clinical syndromes in humans (3, 4). It has recently emerged as an important pathogen in patients with CF, causing severe lung disease (5, 6), and infection with *M. abscessus* is considered a relative contraindication to lung transplantation (7). Moreover, despite cross-infection prevention measures, transmission of multidrug-resistant NTM between patients with CF still occurs (8).

Treatment options are limited because first, *M. abscessus* has a complex cell wall that produces intrinsic resistance to a variety of antibiotics (9, 10); second, antibiotics need to penetrate the macrophage reservoir of the organism; and third, because *M. abscessus* can form biofilms (1, 11). *M. abscessus* is resistant to standard antituberculous antibiotics. Cefoxitin, clarithromycin, and amikacin appear to be active *in vitro*, but these agents show relatively poor efficacy in treating clinical disease (1, 11). New antibiotic approaches to the treatment of *M. abscessus* are urgently needed.

Iron (Fe) is essential for the growth of most microorganisms, including *M. abscessus* (12). Our lab and others have shown that *M. tuberculosis* replicating in human macrophages can acquire Fe bound to transferrin or lactoferrin and from exogenous sources (13–16). Fe is an important component of enzymes involved in critical cellular functions such as DNA synthesis (17), general metabolism, and oxidative stress resistance. Furthermore, Fe availability can mediate bacterial virulence and pathogenesis functions (18, 19). Our laboratory and others have shown that control of Fe availability or interference with Fe uptake could inhibit the growth of *M. tuberculosis* and other bacteria, regardless of whether they are growing extracellularly or within human macrophages (18–21). In addition, we and others have recently reported that the virulence of some bacteria, including mycobacteria, is increased by greater availability of Fe in animal models (21–24). Therefore, targeting of *M. abscessus* Fe metabolism is a promising approach for novel therapy.

Gallium (Ga) shows many similarities to Fe (17), and Ga in the form of $Ga(NO_3)_3$ is an FDA-approved drug for the treatment of hypercalcemia of malignancy (25). In biological systems, Fe^{3+} can be reduced to Fe^{2+}, making it useful as a metal cofactor to facilitate electron transfer by a variety of enzymes. In spite of its similarity to Fe^{3+}, Ga^{3+} does not undergo reduction under physiologic conditions (17). These characteristic makes Ga^{3+} an

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Address correspondence to Bradley E. Britigan, bradley.britigan@unmc.edu.
M.Y.A. and B.L.S. contributed equally to this work.

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attractive "Trojan-horse" metabolic inhibitor since it can both compete with Fe for acquisition and inhibit Fe-dependent enzymes if it is substituted for Fe^{3+} in their active sites. For example, we recently demonstrated that Ga disrupts Fe metabolism and inhibits key Fe-containing regulatory enzymes such as ribonucleotide reductases and aconitase (21). Our lab and others showed the ability of Ga to inhibit the growth of a variety of human pathogens, including several mycobacterial species (17, 19–21, 26–28). In addition, Ga has been active in murine models of several bacterial infections, including *M. tuberculosis* (21).

In the present work, we show that Ga exhibits potent *M. abscessus* growth-inhibitory capacity. We also extended our research to show the effectiveness of different Ga compounds against antibiotic-resistant clinical isolates obtained from CF patients.

MATERIALS AND METHODS

Materials. A reference strain of *M. abscessus* (ATCC 19977) was purchased from the American Type Culture Collection. Clinical isolates of *M. abscessus* subsp. *massiliense* from the University of Washington (MC6067, MC5260, MC5315-1, MC5315-2, MC2638, and MC5597) were acquired by Moira Aitken (University of Washington), characterized, and transferred to our laboratory by material transfer agreement from the Mycobacteria/Nocardia Laboratory at the University of Texas Health Science Center, Tyler, TX (6, 29). Additional unrelated clinical isolates of *M. abscessus* were provided by Paul Fey, associate director of the Clinical Pathology/Microbiology Laboratory at Nebraska Medicine, Omaha, NE.

Sodium citrate, dehydrate, citric acid monohydrate, and ferric nitrate $Fe(NO_3)_3$ were purchased from Fisher Scientific, Fair Lawn, NJ. D-Malic acid, L-malic acid, sodium succinate (dibasic) hexahydrate, oxalic acid, and deferoxamine mesylate salt were purchased from Sigma-Aldrich, St. Louis, MO. Gallium nitrate hydrate [$Ga(NO_3)_3$] was obtained from Acros Organics, Pittsburgh, PA; and pyridoxal isonicotinoyl hydrazine (PIH) was obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Chelex 100 resin was purchased from Bio-Rad Laboratories, Richmond, CA. Gallium maltolate was provided by Aridis Pharmaceuticals, San Jose, CA; and gallium protoporphyrin was purchased from Frontier Scientific, Logan, UT. Apotransferrin was purchased from Athens Research, and RPMI 1640, L-glutamine, HEPES, sodium pyruvate, and fetal bovine serum were obtained from HyClone, Logan, UT.

Gallium chelates. Gallium chelates (sodium citrate, citric acid, D- and L-malic acid, sodium succinate, and oxalic acid) were prepared with a 1.0:1.5 molar ratio of gallium nitrate plus chelate in Fe-free water and mixed for at least 24 h at 4°C as described previously (30). Gallium maltolate, gallium protoporphyrin, and gallium PIH were prepared in a 1:1 molar ratio as described previously (31). All solutions were prepared with water treated with BT Chelex 100 resin at 10 g/liter of water. Gallium transferrin was prepared as previously described (31, 32). All reagents were verified spectroscopically. Briefly, apotransferrin was combined at a 1:3 molar ratio with gallium nitrate in an acetic acid sodium chloride solution, buffered to pH 7.4 with $NaHCO_3$, and mixed for 6 h at 4°C. The compound was dialyzed in phosphate-buffered saline (PBS)–1 mM $NaHCO_3$ with Chelex gel to capture nonbound gallium.

Fe-free 7H9 medium (32) was made in our laboratory by using the standard formulation but without ferric ammonium citrate. It has a final concentration of 0.005% (vol/vol) glycerol, no bovine albumin fraction V-dextrose-catalase, and 0.5% (vol/vol) Tween 80. The Fe content was determined to be 2 μM with the standard ferrozine assay (33).

***In vitro* growth inhibition in broth culture.** Bacteria were grown at 35°C with shaking for 3 days in Fe-free 7H9 medium. The bacterial suspensions were diluted to yield an optical density at 600 nm (OD_{600}) between 0.020 and 0.200. This value was multiplied by a conversion factor based on the CFU counts of bacterial suspensions with an OD_{600} of 1.0, to give the concentration of bacteria at 10^6/ml. A final bacterial concentration of 0.25×10^6/ml was used for all experiments. Outside wells were

TABLE 1 Effects of different concentrations of $Ga(NO_3)_3$ on *in vitro* growth of *M. abscessus* ATCC 19977 and clinical isolates

$Ga(NO_3)_3$ concn (mM)	Normalized mean OD_{600} ± SEM									
	ATCC 19977	MC6067[a]	MC2638	MC5260	MC5315-1	MC5315-2[a]	MC5597[a]	UNMC-1362	UNMC-1374	UNMC-1423	UNMC-1477
2	0.90 ± 0.08	1.08 ± 0.00	0.80 ± 0.03	1.01 ± 0.00	0.79 ± 0.01	1.27 ± 0.00	0.90 ± 0.02	0.55 ± 0.04	0.44 ± 0.05	1.06 ± 0.10	0.96 ± 0.11
10	0.76 ± 0.06	0.70 ± 0.04	0.70 ± 0.01	1.04 ± 0.01	0.71 ± 0.02	0.63 ± 0.02	0.28 ± 0.01	0.27 ± 0.02	0.37 ± 0.13	0.65 ± 0.02	0.78 ± 0.04
15	0.59 ± 0.02	0.49 ± 0.03	0.66 ± 0.00	0.90 ± 0.01	0.69 ± 0.05	0.43 ± 0.02	0.01 ± 0.01	0.28 ± 0.01	0.41 ± 0.18	0.58 ± 0.01	0.67 ± 0.08
20	0.53 ± 0.02	0.30 ± 0.04	0.67 ± 0.02	0.84 ± 0.06	0.62 ± 0.04	0.05 ± 0.01	0.01 ± 0.01	0.25 ± 0.05	0.22 ± 0.05	0.56 ± 0.19	0.57 ± 0.05
25	0.69 ± 0.05	0.10 ± 0.00	0.52 ± 0.01	0.56 ± 0.02	0.63 ± 0.03	0.04 ± 0.03	0.02 ± 0.01	0.21 ± 0.01	0.28 ± 0.02	0.40 ± 0.03	0.43 ± 0.03
30	0.35 ± 0.02	0.02 ± 0.00	0.51 ± 0.01	0.62 ± 0.06	0.48 ± 0.03	0.03 ± 0.01	0.01 ± 0.00	0.25 ± 0.05	0.22 ± 0.02	0.34 ± 0.01	0.41 ± 0.03
40	0.35 ± 0.03	0.00 ± 0.01	0.51 ± 0.01	0.52 ± 0.00	0.44 ± 0.06	0.00 ± 0.01	0.01 ± 0.00	0.28 ± 0.01	0.23 ± 0.03	0.29 ± 0.01	0.29 ± 0.01
50	0.45 ± 0.03	0.02 ± 0.01	0.48 ± 0.03	0.61 ± 0.03	0.17 ± 0.02	0.00 ± 0.00	0.02 ± 0.02	0.23 ± 0.01	0.21 ± 0.01	0.14 ± 0.01	0.42 ± 0.06

[a] $P < 0.05$.

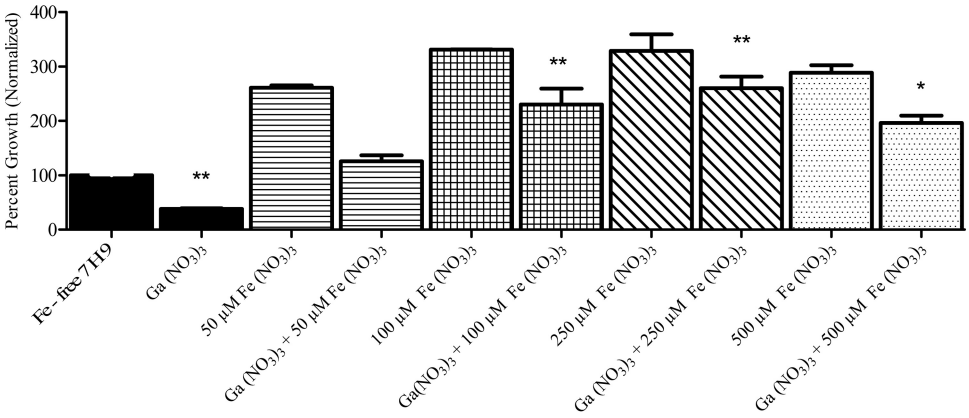

FIG 1 $Ga(NO_3)_3$-mediated growth inhibition is reversed by Fe. Strain ATCC 19977 was cultured in the presence of 50 μM $Ga(NO_3)_3$ and increasing concentrations of $Fe(NO_3)_3$ for up to 7 days. Addition of equimolar or greater amounts of Fe relative to Ga increased *M. abscessus* growth significantly. *, $P < 0.05$; **, $P < 0.01$; $n = 3$ replicates.

filled with deionized water to maintain moisture and growth conditions. Gallium chelates were added to the wells to achieved the desired concentrations. Plates were incubated statically at 37°C for up to 7 days, with OD_{600} readings taken on a daily basis with a microplate reader (Synergy H1; BioTek, Winooski, VT) to measure turbidity, indicating growth. Blank values were subtracted and adjusted "to medium." OD values of growth in wells containing each gallium compound were divided by the OD values of the drug-free control. Controls included non-gallium-bound chelates (showed no effect on bacterial growth), gallium nitrate as an internal control, and Fe-free 7H9 medium as reagent blanks. Gallium protoporphyrin assays included a nonbacterial control for color at each concentration tested.

***In vitro* inhibition of *M. abscessus* growth in the THP-1 macrophage cell line.** THP-1 cells (human monocytic cell line; ATCC TIB-202) were maintained in RPMI 1640–10 mM HEPES–2 mM L-glutamine–1 mM sodium pyruvate–10% fetal bovine serum. Cells were cultured and transformed into functionally mature macrophages with phagocytic activity in the presence of 15 ng/ml TPA (12-*O*-tetradecanoylphorbol-13 acetate; Cell Signaling) overnight at 37°C in 5% CO_2 as described elsewhere (34). Cells were then washed, infected with bacteria at a multiplicity of infection of 10 THP-1 cells to 1 bacterium for 1 h at 37°C under 5% CO_2. The desired concentrations of gallium chelates were then added to the cell cultures, following which they were incubated at 37°C under 5% CO_2. At defined times, the cells were washed and harvested for of *M. abscessus* CFU counting. Potential toxicity of compounds for the THP-1 cells was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (35).

CFU counting. All supernatants and first washes were collected from the plates and combined for CFU counting. After centrifugation at 250 × *g* for 7 min, pellets were suspended in chilled, Chelex-treated, deionized, sterile water and vortexed. Adherent cells were washed, and resuspended pellets (in water) were added to the adherent cell wells and incubated on ice for 10 min. Lysis buffer was then added as described previously (20). Contents were collected and centrifuged at 14,000 × *g* for 15 min. Pellets were resuspended in sterile PBS and plated on tryptic soy agar plates. Colonies were counted 3 to 5 days after plating. Accepted CFU counts were in the range of 15 to 200 colonies in the plating area.

Statistical analysis of data. Means and standard deviations were calculated from independent experiments. Statistical analysis was done with GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA). Differences between three or more means were determined by one- and two-way analyses of variance with Bonferroni *post hoc* tests. Error bars represent standard errors of the means (SEM). All statistical analyses were considered significant at a *P* value of <0.05.

RESULTS

Gallium inhibits growth of ATCC 19977 and clinical isolates of *M. abscessus*. Like most bacteria, including other mycobacterial species, *M. abscessus* requires Fe for growth and metabolism (12). In the present work, we tested the *in vitro* efficacy of Ga against an *M. abscessus* strain obtained from ATCC, as well as clinical isolates obtained from two different institutions.

As shown in Table 1, when *M. abscessus* ATCC 19977 and the clinical isolates were grown in the presence of increasing concentrations of $Ga(NO_3)_3$, significant inhibition ($P < 0.05$) of the growth of the *M. abscessus* strains was observed (Table 1). Each of the clinical isolates from an outbreak among CF patients at the University of Washington varied somewhat in their level of inhibition by Ga. For example, growth of *M. abscessus* MC5597 was completely inhibited by Ga at 4 μM (1.02 μg/ml), whereas *M. abscessus* MC5260 was still able to grow somewhat at Ga concentrations of up to 12 μM (3.06 μg/ml) (Table 1). Clinical isolates obtained from the University of Nebraska Medical Center (UNMC) also demonstrated susceptibility to Ga, with somewhat less variation in their dose-response curves than the Washington isolates (Table 1). Almost 70% of the clinical isolates were inhibited more by Ga than was the reference lab strain (ATCC 19977).

The growth-inhibitory effect of Ga is reversed by exogenous Fe. Previous work with other bacterial species found that the growth-inhibitory effect of Ga relates to disruption of bacterial Fe uptake/utilization, as evidenced by reversal of Ga-mediated growth inhibition by increasing concentrations of extracellular Fe (20). We hypothesized that this would be the case with *M. abscessus* as well. We measured growth inhibition of *M. abscessus* in the presence of increasing concentrations of Fe $(NO_3)_3$ (Fig. 1) and found that addition of Fe to the medium reversed the growth-inhibitory effects of Ga. The addition of an equimolar or greater ratio of $Fe(NO_3)_3$ to Ga was required to restore growth to control levels (Fig. 1). Doubling the molar concentration of Fe further enhanced *M. abscessus* growth. However, the inhibitory effect of Ga was still noticeable even after the addition of a 10× molar concentration of Fe (Fig. 1). This observation is similar to our previous findings with *P. aeruginosa* (26). These data are consistent with the idea that the growth-inhibitory effect of Ga on *M. abscessus* is

TABLE 2 Effects of different Ga chelates at 10 μM on *in vitro* growth of *M. abscessus* ATCC 19977 and clinical isolates

Gallium chelate	Normalized mean OD_{600} ± SEM						
	ATCC 19977	MC6067	MC2638	MC5260	MC5315-1	MC5315-2	MC5597
Gallium nitrate	0.72 ± 0.06	0.89 ± 0.05	0.76 ± 0.03	0.80 ± 0.05	0.63 ± 0.05	0.79 ± 0.07	0.58 ± 0.08
Gallium citrate	0.99 ± 0.06	1.35 ± 0.18	0.99 ± 0.07	1.06 ± 0.04	1.31 ± 0.04	1.08 ± 0.05	1.09 ± 0.01
Gallium citric acid	1.00 ± 0.01	0.58 ± 0.03	0.45 ± 0.00	0.49 ± 0.02[a]	0.76 ± 0.00	0.15 ± 0.029[a]	0.01 ± 0.00[a]
Gallium D-malic	0.92 ± 0.04	0.79 ± 0.02	0.76 ± 0.02	0.86 ± 0.02	0.78 ± 0.02	0.04 ± 0.00[a]	0.84 ± 0.17
Gallium L-malic	1.21 ± 0.14	0.72 ± 0.01	0.81 ± 0.01	0.79 ± 0.01	1.09 ± 0.07	0.13 ± 0.02[a]	0.81 ± 0.16
Gallium maltolate	0.69 ± 0.00	0.35 ± 0.01	0.32 ± 0.03	0.35 ± 0.02	0.43 ± 0.00	0.56 ± 0.02	1.42 ± 0.08
Gallium oxalic	1.12 ± 0.01	0.79 ± 0.04	0.99 ± 0.02	0.82 ± 0.01	0.78 ± 0.06	0.01 ± 0.00[a]	0.91 ± 0.19
Gallium succinic	0.87 ± 0.07	0.86 ± 0.04	0.78 ± 0.01	0.85 ± 0.02	0.53 ± 0.06	0.63 ± 0.05	1.16 ± 0.04
Gallium transferrin	0.84 ± 0.02	0.66 ± 0.02	0.51 ± 0.01	0.79 ± 0.09	0.57 ± 0.03	0.60 ± 0.05[a]	0.02 ± 0.01[a]
Gallium PIH	0.73 ± 0.06	0.89 ± 0.08	0.82 ± 0.02	0.93 ± 0.03	0.59 ± 0.03	1.06 ± 0.04	1.27 ± 0.02

[a] $P < 0.01$ compared to gallium nitrate.

mediated by interference with *M. abscessus* Fe metabolism, as we and other have seen with other bacterial species (19–21).

Inhibition exhibited by different Ga compounds. We sought to determine if other forms of Ga might possess a greater ability than $Ga(NO_3)_3$ to inhibit *M. abscessus* growth. A concentration of 10 μM Ga was used, which does not result in maximal growth inhibition for $Ga(NO_3)_3$ to more readily allow identification of Ga formulations with greater activity than $Ga(NO_3)_3$. The other Ga salts tested included Ga-citrate, Ga-citric acid, Ga–D-malic acid, Ga–L-malic acid, Ga-maltolate, Ga-oxalic acid, and Ga-succinic acid (Table 2), and they were tested against ATCC 19977 and clinical isolates of *M. abscessus*. With the exception of Ga-citrate, most of the Ga compounds exhibited approximately equivalent activity against *M. abscessus* relative to that of $Ga(NO_3)_3$ over 7 days of culture (Table 2). Ga-maltolate exhibited a somewhat higher inhibitory effect (Table 2). Certain clinical isolates (MC5597) were inhibited more by some Ga compounds than others (Table 2). Thus, although some modest differences were observed, none of these chelates demonstrated a major difference in potency from that of $Ga(NO_3)_3$.

We also tested both ATCC 19977 and clinical isolates against a set of Ga compounds in which the Ga was chelated to larger molecule, protoporphyrin (Fig. 2). As shown in Fig. 2, Ga-protoporphyrin was more active. It completely and significantly ($P < 0.001$) inhibited all strains of *M. abscessus* at 10 μM Ga (Fig. 2).

Ga-protoporphyrin inhibits clinical isolates of *M. abscessus*. To further define the pronounced growth-inhibitory action of Ga-protoporphyrin, we titrated Ga-protoporphyrin to lower concentrations with clinical isolate MC2638 (Fig. 3A). Ga-protoporphyrin completely inhibited *M. abscessus* at lower concentrations (1 μM) and showed growth-inhibitory activity at a concentration as low as 0.01 μM. Ga-protoporphyrin was about eight times as potent as $Ga(NO_3)_3$ (Fig. 3A). Similarly, Ga-protoporphyrin inhibited the UNMC-1362 *M. abscessus* clinical isolate, as shown in Fig. 3B. Although not shown, similar inhibitory effects were seen with other UNMC *M. abscessus* clinical isolates. In addition, protoporphyrin alone did not show any inhibitory effect on bacteria (Fig. 3A). This demonstrates that the effect is specific for the Ga chelate and not due to the additive effects of Ga and protoporphyrin.

Ga inhibits *M. abscessus* growth inside human macrophages. One of the challenges in the treatment of *M. abscessus* is that the antibiotic must possess the ability to effectively penetrate and inhibit the bacteria growing within host macrophages. Therefore, the effectiveness of a drug against *M. abscessus* in broth medium does not necessarily correlate with its efficacy against intracellular

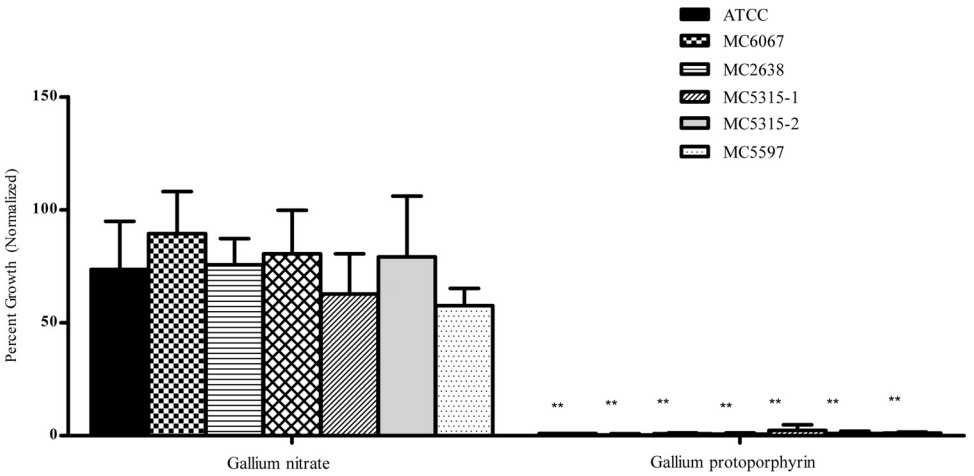

FIG 2 Ga-protoporphyrin shows the greatest inhibition among Ga chelates. *M. abscessus* strains were cultured in the presence of 10 μM Ga-protoporphyrin for up to 7 days. Results show inhibition of all strains normalized to the control. Statistical analysis was done by comparing growth inhibition to that observed with $Ga(NO_3)_3$. **, $P < 0.001$; $n = 3$ replicates.

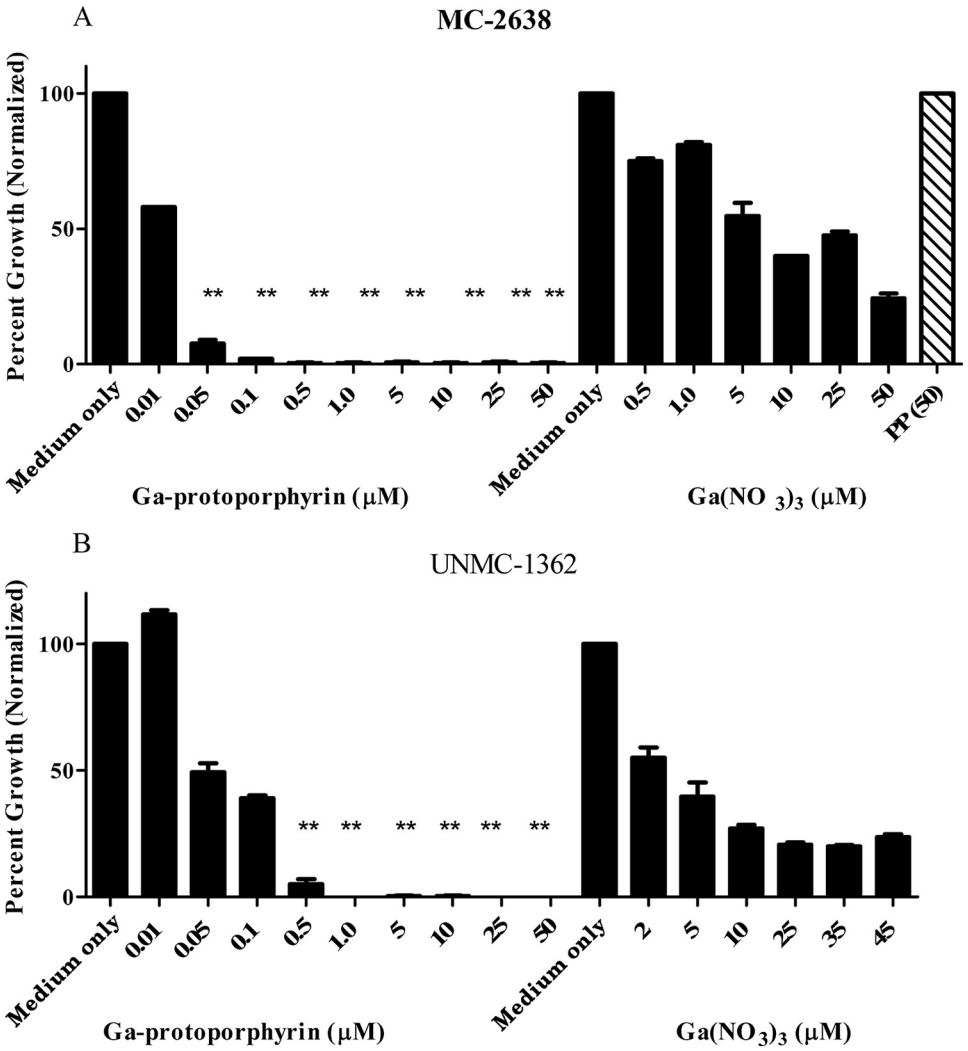

FIG 3 Ga-protoporphyrin inhibits clinical isolates of *M. abscessus*. Clinical isolates MC2638 (A) and UNMC-1362 (B) were cultured in the presence of different concentrations of Ga-protoporphyrin and $Ga(NO_3)_3$ for up to 7 days. Results show inhibition by each compound normalized to the control. PP(50) shows bacterial growth in the presence of protoporphyrin only at 50 μM. Statistical analysis was done by comparing Ga-protoporphyrin to $Ga(NO_3)_3$. **, $P < 0.001$; $n = 3$ replicates.

M. abscessus (36). Accordingly, we investigated whether a similar pattern of growth inhibition would be observed with Ga against *M. abscessus* growing within human macrophages. In order to eliminate the potential confounding effect of different human donors that would occur with primary macrophages, we used TPA-activated THP-1 cells as a model for macrophages. As shown in Fig. 4, $Ga(NO_3)_3$ significantly ($P < 0.001$) inhibited *M. abscessus* growth inside THP-1 cells for up to 6 days, as determined by CFU counting (Fig. 4A). When normalized to the control, $Ga(NO_3)_3$ inhibited more than 90% of *M. abscessus* intracellular growth over 6 days of culture (Fig. 4B).

Ga-protoporphyrin inhibits *M. abscessus* growth inside human macrophages. Similarly, we investigated the inhibitory effect of Ga-protoporphyrin against *M. abscessus*-infected THP-1 cells. As shown in Fig. 5, Ga-protoporphyrin significantly inhibited *M. abscessus* growth inside infected human THP-1 cells compared to the untreated control and to $Ga(NO_3)_3$-treated cells (Fig. 5A). When normalized to the control, 1 μM Ga-protoporphyrin inhibited intracellular *M. abscessus* growth to a level significantly greater than 25 μM $Ga(NO_3)_3$ (Fig. 5B). No significant toxic effects of Ga-protoporphyrin against THP-1 cells were shown at the concentrations used in this experiment (Fig. 5C).

We next expanded the study to include one of the more resistant (Table 2) clinical isolates of *M. abscessus*, MC2638. As shown in Fig. 6A, consistent with observations in broth culture, MC2638 required higher concentrations of $Ga(NO_3)_3$ to inhibit intracellular growth than other *M. abscessus* strains. Nevertheless, when the values were normalized to those of the control, gradual and concentration-dependent inhibition of MC2638 was seen (Fig. 6B).

When MC2638 was cultured in Ga-protoporphyrin for up to 6 days, significant inhibition was seen at 50 μM compared to that obtained with the control and $Ga(NO_3)_3$ treatments (Fig. 7A). When normalized to the control, Ga-protoporphyrin exhibited about 90% inhibition of MC2638 at 6 days of culture and was significantly more effective than $Ga(NO_3)_3$ (Fig. 7B).

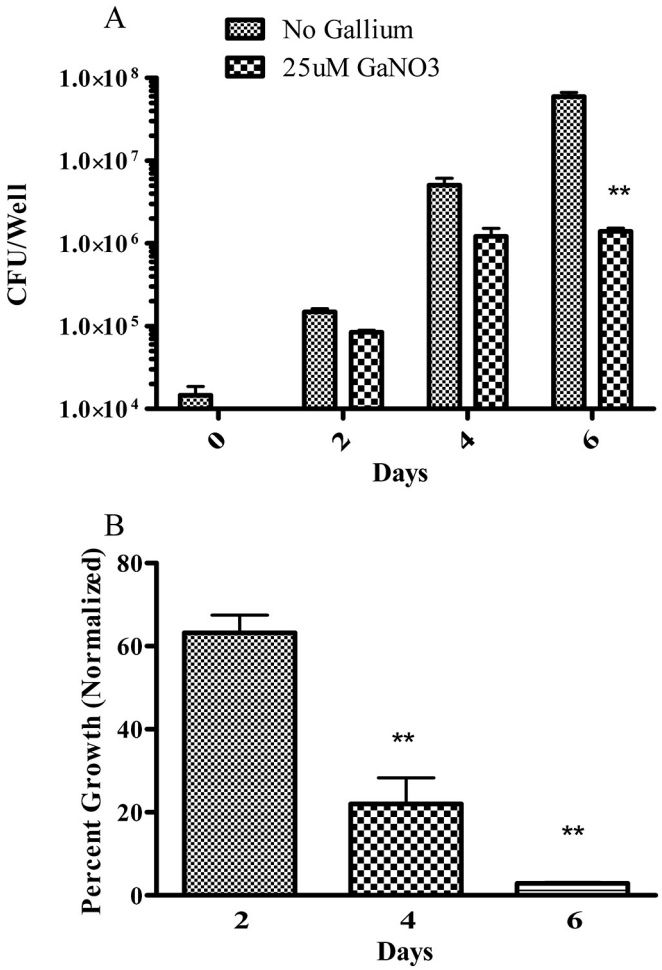

FIG 4 $Ga(NO_3)_3$ inhibits intracellular *M. abscessus* growth. THP-1 cells were TPA activated into macrophage-like cells. Cells were then infected with *M. abscessus* ATCC 19977 and cultured for up to 6 days with or without 25 μM $Ga(NO_3)_3$. Panel A shows results expressed in CFU counts, and panel B shows results normalized to the control (no Ga). Growth of intracellular *M. abscessus* was significantly inhibited in the presence of 25 μM $Ga(NO_3)_3$. **, $P < 0.01$; $n = 3$ replicates.

DISCUSSION

Most recent antibacterial drugs advances have been achieved by modify existing classes of drugs that inhibit critical bacterial targets. Although this is a valid approach, increasing resistance of clinically important pathogens to known classes of antibiotics has made this approach less productive. Therefore, the search for new antibacterial agents and targets is becoming an urgent necessity for the health care community.

M. abscessus is a rapidly growing mycobacterium that is capable of causing human disease, particularly involving the lungs or skin (3, 4). It has recently emerged as an important pathogen in patients with CF, resulting in progressive lung destruction and death (7). *M. abscessus* has a complex cell wall that makes it resistant to many antibiotics. In addition, since *M. abscessus* grows and replicates in macrophages, antibiotics must successfully penetrate this niche to be effective. *M. abscessus* infections respond poorly to currently available antibiotics, resulting in poor clinical outcomes (10, 36). Therefore, new antimicrobial agents and new targets for this organism are needed.

Iron is essential for the growth of most bacteria, including *M. abscessus*, because of the role of Fe in enzymes involved in bacterial DNA synthesis and other critical biochemical functions (17). Iron is also involved in bacterial virulence and pathogenesis (18, 19). Therefore, Fe metabolism and the Fe uptake pathway are potential therapeutic targets for new antibiotics (37–39).

Our group and others have previously shown that Ga disrupts bacterial Fe metabolism and inhibits the growth multiple species of bacteria, regardless of whether they are growing extracellularly (e.g., *P. aeruginosa*) or within human macrophages (e.g., *M. tuberculosis*) (20, 21, 26). Ga has proven to be an effective antimicrobial agent both *in vitro* and in animal models (21). Adding to that, it has been shown to be safe in humans and generally well tolerated. Ga is already a FDA-approved drug for the treatment of hypercalcemia of malignancy.

We have previously shown that Ga-nitrate exhibits potent antimicrobial activity against *M. tuberculosis* and other bacteria that grow intra- or extracellularly (20, 26, 27). This appears to be related to the ability of Ga to disrupt various aspects of bacterial Fe metabolism. The CF pathogen, *P. aeruginosa*, has been found to extensively evolve during infection, and evolution can affect Fe uptake systems (40–42). Despite this, most CF *P. aeruginosa* isolates retain sensitivity to Ga (26, 43–45). The fact that even highly evolved clinical isolates remain Ga-sensitive led us to hypothesize that CF *M. abscessus* isolates would also be inhibited by Gallium compounds.

In this work, we sought to test the potential of Ga-based therapy against *M. abscessus*. Different Ga compounds and chelates were tested against ATCC 19977, as well as clinical isolates of *M. abscessus*. Initial work found that $Ga(NO_3)_3$, the FDA approved form of the Ga, showed the ability to inhibit both lab strains and clinical isolates of *M. abscessus*. The susceptibility to $Ga(NO_3)_3$ varied among the *M. abscessus* strains. Nevertheless, growth inhibition was detected at Ga concentrations well within those achievable in serum and tissues with administration of $Ga(NO_3)_3$ (21). Intravenous $Ga(NO_3)_3$ for treating hypercalcemia leads to steady-state plasma levels of 12 to 32 μM Ga (46, 47). Bolus intravenous infusions yield peak serum levels of 140 to 700 μM (48, 49).

Consistent with work in other bacterial species (21), the Ga effect on *M. abscessus* appears to be mediated through disruption of the bacteria's Fe metabolism. The addition of increasing concentrations of Fe negated the inhibitory effect of Ga, restoring *M. abscessus* growth to control levels.

Although $Ga(NO_3)_3$ is the formulation of Ga currently commercially available, other forms of Ga could prove more active against *M. abscessus*. Therefore, we tested a variety of Ga salts and small-molecule complexes against ATCC 19977 and the clinical *M. abscessus* isolates. A variation in potency was observed, but the modest magnitude is unlikely to be of significance from the standpoint of new drug development. Nevertheless, the result demonstrates that Ga compounds with different solubility properties and chemical characteristics can be effective.

In contrast to results obtained with Ga small-molecule complexes, when Ga compounds in which the Ga was chelated to larger molecules were tested, Ga-protoporphyrin showed a significantly increased ability to inhibit *M. abscessus* growth *in vitro*. Ga-protoporphyrin was previously shown to exhibit antimicrobial activity against *Yersinia enterocolitica*, *Neisseria gonorrhoeae*, *Haemophilus ducreyi*, *Staphylococcus aureus*, *Porphyromonas gingivalis*, and some species of mycobacteria (28, 50, 51). When ex-

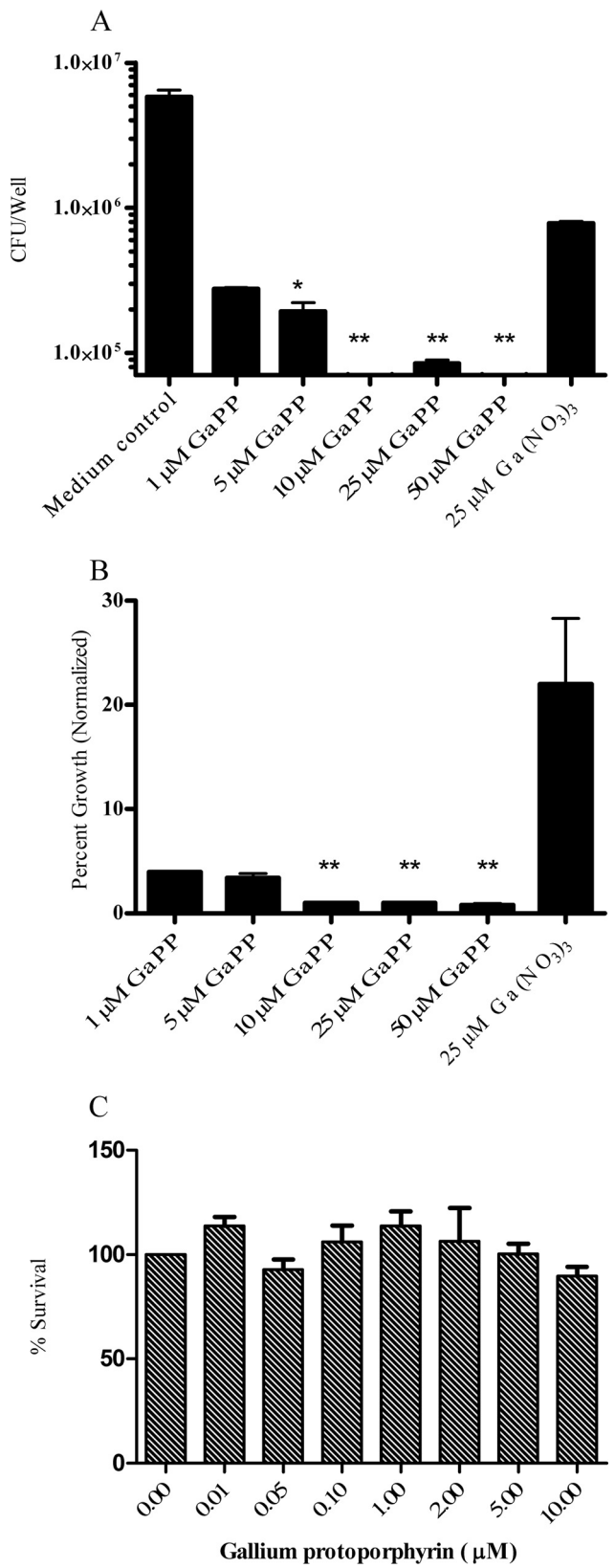

FIG 5 Ga-protoporphyrin inhibits intracellular *M. abscessus* growth. THP-1 cells were transformed and infected with *M. abscessus* ATCC 19977 for 1 h, washed, and cultured for up to 4 days in the presence of different concentrations of Ga-protoporphyrin as described in Materials and Methods. Ga-protoporphyrin significantly inhibited intracellular *M. abscessus* relative to the control (no Ga) or $Ga(NO_3)_3$ treated when expressed as the number of CFU/well (panel A) ($P < 0.001$) or when the data were expressed normalized to the control (no Ga). The time zero CFU count was 2.9×10^4. No toxic effects of Ga-protoporphyrin against THP-1 cells were seen (panel C), as determined by MTT assay. *, $P < 0.01$; **, $P < 0.001$; $n = 4$ replicates.

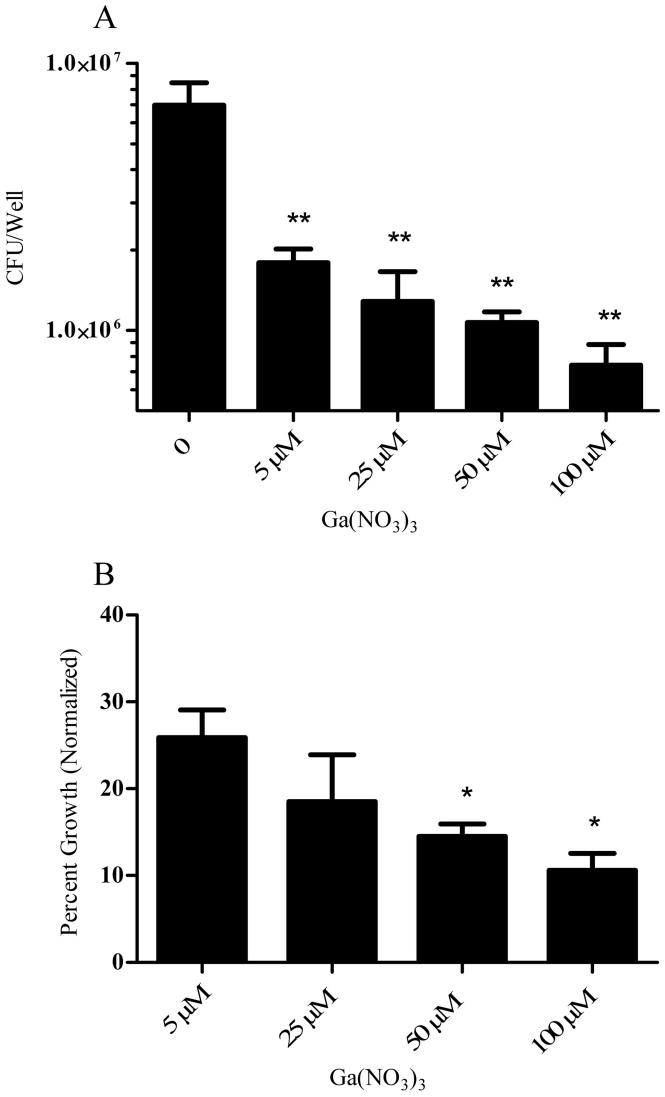

FIG 6 $Ga(NO_3)_3$ inhibits intracellular growth of clinical isolate MC2638 at higher concentrations. THP-1 cells were transformed and infected with clinical isolate MC2638 for 1 h, washed, and cultured for up to 6 days in the presence of different concentrations of $Ga(NO_3)_3$ as described in Materials and Methods. $Ga(NO_3)_3$ significantly inhibited intracellular MC2638 (**, $P < 0.001$) as measured by CFU counting (A) or when results were normalized to the control (B) (*, $P < 0.05$; $n = 4$ replicates). The time zero CFU count was 2.2×10^4.

amined, this activity was in excess of that seen with other Ga chelates, consistent with our results with *M. abscessus*.

M. abscessus grows and replicates within human macrophages during infection (11). In order for an antibiotic to be effective against mycobacterial species *in vivo*, it must be able to enter the macrophage in sufficient quantities to inhibit mycobacterial growth. As we had previously reported with *M. tuberculosis*,

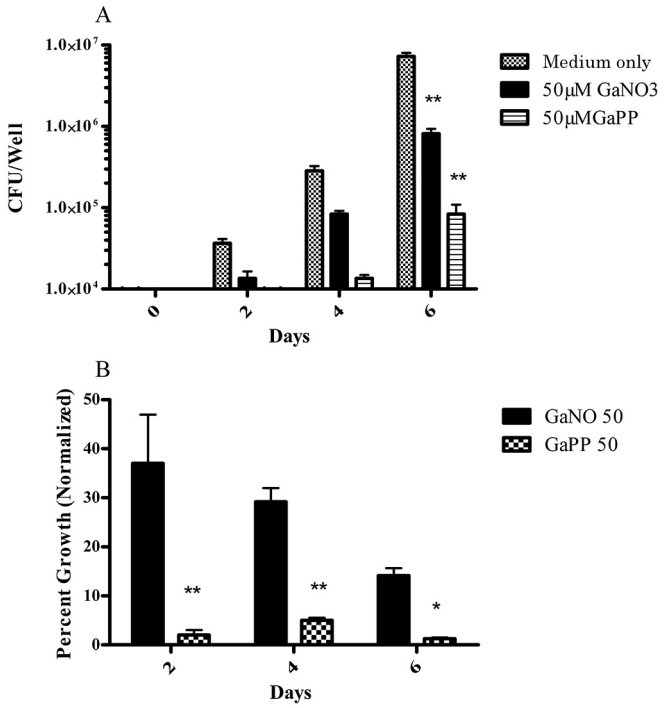

FIG 7 Ga-protoporphyrin inhibits intracellular growth of clinical isolate MC2638. THP-1 cells were transformed and infected with clinical isolate MC2638 for 1 h, washed, and cultured for up to 6 days in the presence of different concentrations of Ga-protoporphyrin as described in Materials and Methods. Ga-protoporphyrin significantly inhibited intracellular MC2638 (**, $P < 0.001$) as determined by CFU counting (A) or when data were normalized to the control (B) (*, $P < 0.05$; $n = 4$ replicates).

$Ga(NO_3)_3$ inhibited intracellular *M. abscessus* growth with THP-1 cells as the macrophage model. As was observed in *in vitro* growth in bacteriologic medium, Ga-protoporphyrin demonstrated greater potency against *M. abscessus* growing within THP-1 cells than $Ga(NO_3)_3$ did.

In summary, we and others have previously reported that $Ga(NO_3)_3$ possesses *in vitro* antimicrobial activity against *M. tuberculosis* and other mycobacterial species (21, 51). $Ga(NO_3)_3$ was also shown to exhibit efficacy in a murine model of tuberculosis. We found that $Ga(NO_3)_3$ exhibits similar activity *in vitro* against *M. abscessus*, a pathogen of growing importance in patients with CF. Our studies confirm that Ga-protoporphyrin appears to possess greater *in vitro* antimicrobial against *M. abscessus*, consistent with findings on other mycobacteria. Although Ga-protoporphyrin exhibited greater *in vitro* activity than $Ga(NO_3)_3$, the potential toxicity of Ga-protoporphyrin has not been extensively studied, whereas the toxicity profile of $Ga(NO_3)_3$ has been more clearly defined, at least in the dosing regimen used in the use of the drug for hypercalcemia of malignancy. Interestingly, a recent small phase 1 trial of parenteral $Ga(NO_3)_3$ in CF patients infected with *P. aeruginosa* has been recently completed that showed significant improvement in lung function (43–45). The drug was well tolerated. The current work suggests that Ga-based therapy offers the potential for development as a novel therapy against *M. abscessus*. The optimal dosing formulation remains to be defined, and *in vivo* efficacy has yet to be demonstrated.

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