

12-2013

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Olakanmi, Oyebode; Kesavalu, Banurekha; Pasula, Rajamouli; Abdalla, Maher Y.; Schlesinger, Larry S.; and Britigan, Bradley E., "Gallium Nitrate Is Efficacious in Murine Models of Tuberculosis and Inhibits Key Bacterial Fe-Dependent Enzymes" (2013). *U.S. Department of Veterans Affairs Staff Publications*. 87.  
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# Gallium Nitrate Is Efficacious in Murine Models of Tuberculosis and Inhibits Key Bacterial Fe-Dependent Enzymes

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Acquiring iron (Fe) is critical to the metabolism and growth of *Mycobacterium tuberculosis*. Disruption of Fe metabolism is a potential approach for novel antituberculous therapy. Gallium (Ga) has many similarities to Fe. Biological systems are often unable to distinguish Ga<sup>3+</sup> from Fe<sup>3+</sup>. Unlike Fe<sup>3+</sup>, Ga<sup>3+</sup> cannot be physiologically reduced to Ga<sup>2+</sup>. Thus, substituting Ga for Fe in the active site of enzymes may render them nonfunctional. We previously showed that Ga inhibits growth of *M. tuberculosis* in broth and within cultured human macrophages. We now report that Ga(NO<sub>3</sub>)<sub>3</sub> shows efficacy in murine tuberculosis models. BALB/c SCID mice were infected intratracheally with *M. tuberculosis*, following which they received daily intraperitoneal saline, Ga(NO<sub>3</sub>)<sub>3</sub>, or NaNO<sub>3</sub>. All mice receiving saline or NaNO<sub>3</sub> died. All Ga(NO<sub>3</sub>)<sub>3</sub>-treated mice survived. *M. tuberculosis* CFU in the lungs, liver, and spleen of the NaNO<sub>3</sub>-treated or saline-treated mice were significantly higher than those in Ga-treated mice. When BALB/c mice were substituted for BALB/c SCID mice as a chronic (nonlethal) infection model, Ga(NO<sub>3</sub>)<sub>3</sub> treatment significantly decreased lung CFU. To assess the mechanism(s) whereby Ga inhibits bacterial growth, the effect of Ga on *M. tuberculosis* ribonucleotide reductase (RR) (a key enzyme in DNA replication) and aconitase activities was assessed. Ga decreased *M. tuberculosis* RR activity by 50 to 60%, but no additional decrease in RR activity was seen at Ga concentrations that completely inhibited mycobacterial growth. Ga decreased aconitase activity by 90%. Ga(NO<sub>3</sub>)<sub>3</sub> shows efficacy in murine *M. tuberculosis* infection and leads to a decrease in activity of Fe-dependent enzymes. Additional work is warranted to further define Ga's mechanism of action and to optimize delivery forms for possible therapeutic uses in humans.

*Mycobacterium tuberculosis* is an intracellular pathogen of human macrophages. It infects the human respiratory system, causing the clinical entity of tuberculosis (TB) (1). In 2011, *M. tuberculosis* was responsible for approximately 8.7 million infections and 1.4 million deaths worldwide (2). Although effective antibiotic treatment is available for drug-susceptible *M. tuberculosis*, it requires administration of multiple antibiotics for many months. However, antibiotic resistance is becoming an increasing and major challenge in form of multiple-drug-resistant tuberculosis (MDR-TB) and extensively drug-resistant (XDR-TB) infections. Hence, the search for new therapeutic approaches is a priority for the scientific community.

Ferric iron (Fe<sup>3+</sup>) is essential for the growth of most microorganisms, including *M. tuberculosis*, as the metal is utilized as a constituent of enzymes and proteins that are critical to various metabolic and DNA-synthetic pathways (3). Our laboratory and others have shown that disruption of iron metabolism inhibits the growth of *M. tuberculosis* and other bacteria regardless of whether they are growing extracellularly or within human macrophages (4–6). The ability of pathogens to obtain iron from host sources such as transferrin, lactoferrin, ferritin, and heme or other iron-containing proteins is considered to be a key virulence factor (4, 5). In animal models, the pathogenicity and virulence of many pathogens, including *M. tuberculosis* and *Mycobacterium avium*, are considerably enhanced as iron availability is increased in the host (7–9). Clinical data suggest that this is the case with human tuberculosis as well (8, 10). Furthermore, new insights into iron acquisition mechanisms by *M. tuberculosis* are being uncovered (11, 12).

Given the importance of iron acquisition to pathogenesis, bac-

terial iron metabolism is an attractive target for therapeutic drug development. Iron chelation approaches have been attempted but have not yet led to successful therapies (13, 14). We and others have pursued an alternative approach in which gallium (Ga), which has remarkable physicochemical similarity to iron, is used to corrupt the bacterial iron acquisition and utilization process.

Gallium, which exists in nature as Ga<sup>3+</sup>, binds to most ferric iron-binding proteins and chelating agents with an affinity similar to that of iron. However, Ga<sup>3+</sup> is not reducible under physiological conditions, whereas Fe<sup>3+</sup> is readily reduced to Fe<sup>2+</sup> (15). The ability to redox cycle between these two oxidation states is critical to the ability of iron to catalyze biochemical reactions. Thus, if cells take up and insert Ga<sup>3+</sup> into active sites of Fe-dependent enzymes, the enzymes are rendered inactive. This property led to the development and FDA approval of Ga-based therapy for the treatment of hypercalcemia of malignancy, in which Ga inhibits ribonucleotide reductase (RR) activity in malignant cells (15).

We previously demonstrated that the presence of Ga inhibits the *in vitro* growth of *M. tuberculosis*, regardless of whether the mycobacteria are growing extracellularly or within cultured human macrophages (6). A series of studies by our laboratory and

Received 18 July 2013 Returned for modification 24 August 2013

Accepted 17 September 2013

Published ahead of print 23 September 2013

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doi:10.1128/AAC.01543-13

others demonstrated that Ga possesses antimicrobial potency against a variety of human pathogens (3, 5, 6, 16–18). Disruption of bacterial iron acquisition/utilization has been shown to correlate with the ability of Ga to inhibit *M. tuberculosis* growth as well as that of other organisms (3, 5, 6, 16, 17). However, the key aspects of *M. tuberculosis* iron metabolism affected by Ga have not been well defined. Furthermore, whether the *in vitro* antimicrobial activity of Ga against *M. tuberculosis* translates to *in vivo* efficacy has not been examined.

Both delineation of the mechanism of action and demonstration of efficacy *in vivo* are critical next steps in the further development of a Ga-based approach to therapy of tuberculosis. The purpose of the present study was to assess the ability of Ga administration to inhibit the growth of virulent *M. tuberculosis in vivo* by using two separate murine models of pulmonary tuberculosis. In addition, we sought insight into key microbial targets disrupted by Ga therapy.

## MATERIALS AND METHODS

**Materials.** Ga(NO<sub>3</sub>)<sub>3</sub>, sodium citrate, acetate, HEPES, dithiothreitol (DTT), and lysozyme were purchased from Sigma-Aldrich, St. Louis, MO. Lysis buffer (B-PER bacterial protein extraction reagents) was purchased from Fisher Scientific, Pittsburg, PA. Additional reagents and their sources are as follows: RNase A, NDP kinase, and DNase were obtained from Invitrogen, Grand Island, NY; ATP was obtained from Alexis Biochemicals, Farmingdale, NY; herring sperm DNA (0.1 to 3 kb) was obtained from Promega, Madison, WI; Klenow polymerase and a random 6-mer primer were obtained from New England BioLabs, Ipswich, MA; and DE-81 filter discs were obtained from GE Healthcare, Pittsburgh, PA. A biconchonic acid (BCA) kit was purchased from Thermo Fisher Scientific, Rockford, IL.

***Mycobacterium tuberculosis* strains and cultivation.** *Mycobacterium tuberculosis* strains H<sub>37</sub>R<sub>a</sub> (ATCC 25177), H<sub>37</sub>R<sub>v</sub> (ATCC 27294), and Erdman (ATCC 35801) were cultivated on 7H11 agar plates for 10 days. They were then harvested into 7H9 medium containing 10 mM HEPES, to form predominantly single-cell suspensions (19), and used shortly thereafter.

**Infection of mice with *M. tuberculosis*.** BALB/c and BALB/c SCID mice, 6 to 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). BALB/c mice are relatively susceptible to infection with *M. tuberculosis*. Mice were maintained under sterile conditions in microisolation cages in a barrier facility and supplied with sterilized food and water. All animal procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Mice were infected intratracheally (i.t.) with the desired numbers of CFU of either the H<sub>37</sub>R<sub>v</sub> or Erdman strain of *M. tuberculosis* suspended in 50 μl Hanks' balanced salt solution (HBSS) in a class III biohazard safety cabinet and were maintained in the biosafety level 3 (BSL3) animal facility at the University of Cincinnati. Infected mice were then treated daily with 50 μl of phosphate-buffered saline (PBS), Ga nitrate, or sodium nitrate intraperitoneally (i.p.). At specific times following *M. tuberculosis* administration, mice were sacrificed, and the burden of *M. tuberculosis* was determined by counting CFU in lung homogenates. We infected SCID mice with 100 CFU of *M. tuberculosis* (strain H<sub>37</sub>R<sub>v</sub>) bacteria i.t., followed by daily i.p. administration of 10 mg/kg of body weight Ga(NO<sub>3</sub>)<sub>3</sub>, a dosage that was previously shown to be well tolerated (10 mg/kg) (16, 17). Control mice received either saline or a molar equivalent of NO<sub>3</sub><sup>-</sup> (NaNO<sub>3</sub>) to that provided by Ga(NO<sub>3</sub>)<sub>3</sub>, the latter as a control for any possible effects of NO<sub>3</sub><sup>-</sup>. The mice were then observed daily. The concentration of Ga used in this study (10 mg/kg) is in the range of that achievable *in vivo* and found to be safe for human use (3, 20).

**Determination of *M. tuberculosis* CFU in tissue organs.** To assess *M. tuberculosis* numbers in the tissues of the infected mice, at specified time points, lungs, spleen, and liver were removed aseptically, cut into small pieces, and homogenized. Viable *M. tuberculosis* bacteria in the tissue

homogenate were quantified by determining CFU as described in our previous studies (6). Briefly, serial dilutions of tissue homogenates were plated in duplicate onto 7H11 Middlebrook agar in 6-well plates. The inoculated agar plates were dried at room temperature and incubated in a 37°C incubator for up to 3 weeks, at which time colonies were counted. The results were expressed as means ± standard deviations of tissue CFU per whole organ under each experimental condition and for different treatments.

**Human monocyte-derived macrophage preparation.** Peripheral venous blood was obtained from healthy adult volunteers according to a University of Cincinnati College of Medicine-approved institutional review board (IRB) protocol. Mononuclear cells were separated by Ficoll-Hypaque centrifugation and cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 20% autologous human serum for 5 days in Teflon wells in a 5% CO<sub>2</sub> atmosphere at 37°C to allow for differentiation into monocyte-derived macrophages (MDM). MDM monolayers were then maintained in 24- or 6-well culture plates, supplemented with 20% autologous serum, and incubated at 37°C for 1 day prior to incubation with *M. tuberculosis*.

**Preparation of cell extract for the RR assay.** The protocol for preparation of cell extracts was adapted from the original protocol by Jong et al. for the RR assay (21). *M. tuberculosis* strain H<sub>37</sub>R<sub>a</sub> was inoculated into defined Middlebrook 7H9 (D7H9) medium with and without 1 μM ferric ammonium sulfate and with and without Ga(NO<sub>3</sub>)<sub>3</sub> (10 to 100 μM). The bacteria were then incubated overnight at 37°C with shaking, with monitoring of the optical density (OD) (at 600 nm). The suspension was centrifuged at 10,000 × g for 10 min at 4°C and washed twice in buffer I (500 mM, pH 7.2). The resulting pellet was weighed and finally suspended in lysis buffer (B-PER) containing 50 mg/ml lysozyme, 0.2 mg/ml RNase A, DNase, and an antiprotease cocktail at 4 ml/g of wet bacterial pellet. The suspension was rocked for 30 min at room temperature and centrifuged at 12,000 × g for 10 min at 25°C, and the supernatant was decanted. The pellet was homogenized on ice by using a glass homogenizer. The homogenate was centrifuged at 16,000 × g for 20 min at 4°C, and the supernatant was collected. Two hundred microliters of the supernatant was then loaded onto a Sephadex G-25 column that was pre-equilibrated with ice-cold buffer II (50 mM HEPES [pH 7.2], 2 mM DTT) and centrifuged at 1,500 × g for 4 min at 4°C to remove endogenous nucleotides. The flow-through was collected, and the protein concentration was determined by using a BCA kit.

The RR reaction mixture, consisting of 50 mM HEPES (pH 7.2), 18 mM DTT, 12 mM Mg acetate, 6 mM ATP, and 100 pmol [<sup>14</sup>C]CDP (405 mCi/mmol; Moravek, Brea, CA), was prepared in Eppendorf tubes. The reaction was initiated by the addition of 250 μg of flowthrough protein (400 μg), isolated from *M. tuberculosis* as described above, and incubation at 37°C for 30 min. The reaction was terminated in boiling water for 5 min. The substrate [<sup>14</sup>C]CDP was converted to [<sup>14</sup>C]dCDP by the addition of 100 pmol of ribonucleotide reductase for 30 min at 37°C and subsequently to [<sup>14</sup>C]dCTP by the endogenous NDP kinase in the reaction mixture. The mixture was centrifuged at 16,000 × g for 5 min at 25°C to remove the precipitates, and the clear supernatant containing [<sup>14</sup>C]dCTP was used for the DNA polymerase coupling assay.

To serve as the template for the assay, 2 μg herring sperm DNA (0.1 to 3 kb) was mixed with 330 ng of a random 6-mer primer. The labeling mixture contained 90 mM HEPES (pH 6.6), 18 mM DTT, 10 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphate (dNTP), and 5 units of Klenow polymerase. The boiled RR reaction mixture containing [<sup>14</sup>C]dCTP was added to this mixture and incubated at room temperature for 30 min. The reaction was terminated by spotting 40 μl of the mixture onto DE-81 filter discs. The filters were air dried overnight and washed with 5% Na<sub>2</sub>HPO<sub>4</sub> for 5 min by shaking gently on a platform rotator, and the medium was removed. Washing was repeated two more times with 5% Na<sub>2</sub>HPO<sub>4</sub> for 2 min each. The filters were then rinsed with distilled water twice, followed by 95% ethanol once. The paper was dried under a heat lamp. The completely dried filter papers were transferred into a vial with 20 ml Aquasol

for scintillation counting. Two microliters (100 pmol) of [ $^{14}\text{C}$ ]CDP was spotted onto one of the discs for total counts. A sham control that has all other reagents, except the protein lysate, was included in the reaction. This value, representing a random reaction, was subtracted from other counts.

**Aconitase assay.** Bacteria were cultured, collected, and lysed as described above for the RR assay. Aconitase activity was assayed in the resulting cell lysate by according to methods described previously by Kennedy et al., by monitoring the conversion of isocitrate to *cis*-aconitate, as indicated by the increase in the absorbance at 240 nm (22). The assay was performed with 20 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 7.7) containing 250  $\mu\text{M}$  isocitrate.

**Data analysis.** Data were analyzed and statistical tests were performed by using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA). Student's *t* test was employed for analysis of two groups. To determine differences between three or more means, we used one-way analysis of variance (ANOVA) with Bonferroni *post hoc* tests. Error bars represent means  $\pm$  standard errors of the means (SEM). All statistical analyses were considered significant at a *P* value of  $<0.05$ . A *t* test was used to compare survival curves.

## RESULTS

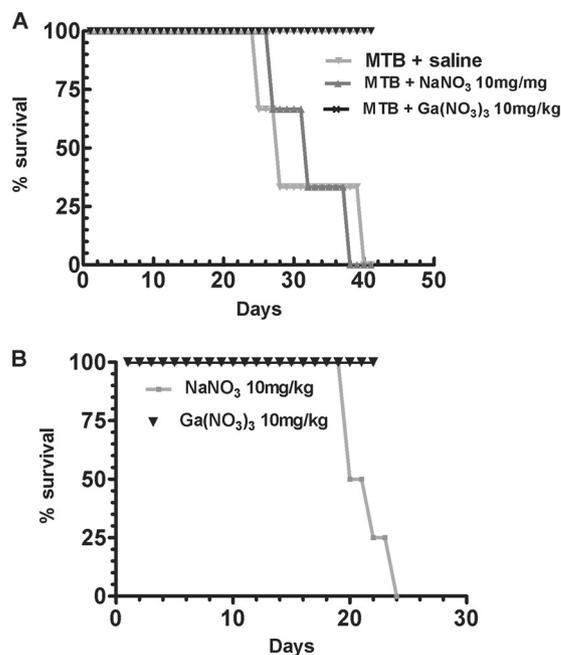
**Gallium is efficacious in a SCID mouse *M. tuberculosis* infection model.** We have shown previously that Ga disrupts iron metabolism and inhibits the growth of *M. tuberculosis* extracellularly or within human macrophages (6). However, a growth-inhibitory effect *in vitro* does not necessarily ensure an effect *in vivo*. Therefore, we sought to determine the effect of Ga on the course of *M. tuberculosis* infection in an established mouse model.

In the first set of experiments, we chose to assess the effectiveness of Ga treatment against *M. tuberculosis* in a murine model in which host defenses were unable to contain the infection: severe combined immunodeficient (SCID) mice. Control mice died after 25 to 40 days postinfection. However, mice receiving  $\text{Ga}(\text{NO}_3)_3$  survived for up to 6 weeks, at which time they were sacrificed (Fig. 1A). The survival rate was significantly higher for Ga-treated mice (100% survival up to 40 days) than for the saline- or nitrite-treated groups ( $P < 0.05$ ).

Given this positive result, we studied the effect of Ga using a higher inoculum of *M. tuberculosis*. When the inoculum of *M. tuberculosis* was increased to  $10^6$  CFU, all control mice died between days 20 and 24. All mice receiving  $\text{Ga}(\text{NO}_3)_3$  remained alive at 28 days (Fig. 1B).

We also studied the tissue burden of *M. tuberculosis* by counting CFU determined at the time of death for the saline- and  $\text{NaNO}_3$ -treated mice or at the time of sacrifice of the  $\text{Ga}(\text{NO}_3)_3$ -treated SCID mice. Ga treatment significantly reduced the number of *M. tuberculosis* CFU in the lungs relative to  $\text{NaNO}_3$  (Fig. 2) or saline (not shown). Compared to  $\text{NaNO}_3$  controls, lung CFU decreased by 89.9% (Fig. 2). In addition, Ga administration also significantly decreased the level of *M. tuberculosis* in other organs, liver (97.7%) and spleen (84.8%), relative to  $\text{NaNO}_3$ .

**Gallium is efficacious in a BALB/c mouse *M. tuberculosis* infection model.** We next assessed the effectiveness of Ga in a murine model that does not lead to lethal infection but rather leads to a stable chronic infection. BALB/c mice were infected with 100 CFU of the virulent Erdman strain of *M. tuberculosis* i.t., followed by daily i.p.  $\text{Ga}(\text{NO}_3)_3$ . Control mice received either saline or  $\text{NaNO}_3$ , the latter at a dose equivalent to the amount of  $\text{NO}_3$  provided by administration of  $\text{Ga}(\text{NO}_3)_3$ . As shown in Fig. 3, in BALB/c mice, *M. tuberculosis* infection led to a chronic nonlethal infection. This resulted in a loss of weight of the animals over time (Fig. 3A). *M. tuberculosis*-infected animals that received Ga exhib-



**FIG 1** Gallium is protective in a SCID mouse TB model. (A) Survival of the mice as a function of time and treatment regimen. Three groups of 6- to 8-week-old BALB/c SCID mice (3 mice/group) were infected with 100 CFU of *M. tuberculosis* (strain  $H_{37}R_v$ ) i.t., following which they received daily i.p. administration of saline, 10 mg/kg  $\text{Ga}(\text{NO}_3)_3$ , or  $\text{NaNO}_3$ . (B) Same experiment as in panel A but using a higher *M. tuberculosis* inoculum. Three groups of 6- to 8-week-old BALB/c SCID mice (4 mice/group) were infected with  $10^6$  CFU of *M. tuberculosis* (strain  $H_{37}R_v$ ) i.t., following which they received daily i.p. administration of 10 mg/kg  $\text{Ga}(\text{NO}_3)_3$  or  $\text{NaNO}_3$ . The figure shows the survival of the mice as a function of time and treatment regimen. The survival rate was significantly higher for Ga-treated mice (100% survival up to 40 days) than for saline- or nitrite-treated groups ( $P < 0.05$ ).

ited less weight loss than mice receiving saline ( $P < 0.05$ ) (Fig. 3A). Compared to the saline control, initiation of Ga treatment significantly reduced *M. tuberculosis* growth in lung tissues (53%) at 30 days as well as in spleen (79%) ( $P < 0.05$ ) (Fig. 3B and C). Ga reduced *M. tuberculosis* growth in the liver (49%), but the difference did not reach significance ( $P > 0.05$ ) (Fig. 3D). When Ga treatment was stopped at 15 days postinfection, the bacteria resumed growth, as shown by an increase in lung CFU (Fig. 3B).

**Ga inhibits *M. tuberculosis* ribonucleotide reductase activity.** Having obtained evidence of the efficacy of Ga *in vivo*, we sought to obtain additional insights into the mechanism(s) by which Ga exhibits antimycobacterial activity. Previous work showed that Ga-mediated inhibition of *M. tuberculosis* growth involved disruption of bacterial iron metabolism. The iron-dependent enzyme ribonucleotide reductase (RR) is essential for DNA synthesis (23). *M. tuberculosis* contains class Ib and class II ribonucleotide reductases (24). Previous studies of eukaryotic cells found that a major mechanism whereby Ga inhibits cell proliferation is by inhibiting cellular RR activity (25). We therefore assessed the specific effect of Ga on *M. tuberculosis* RR activity.

We treated *M. tuberculosis*  $H_{37}R_a$  with different concentrations of Ga for 24 h. Inhibition of RR was seen at all levels of Ga treatment. Inhibition increased significantly when the Ga concentration was doubled from 10 to 20  $\mu\text{M}$  (Fig. 4). However, further inhibition was not seen when the Ga dose was increased to 50 or

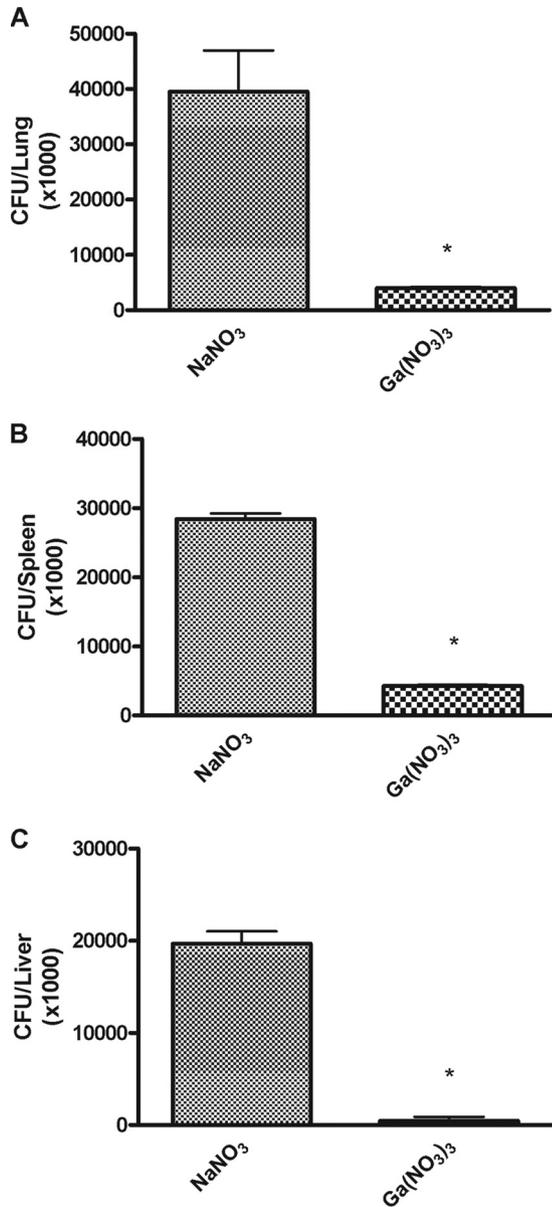


FIG 2 Gallium decreases *M. tuberculosis* growth in lungs and dissemination to different tissues in a SCID mouse TB model. *M. tuberculosis* growth following i.t. inoculation was determined as a function of NaNO<sub>3</sub> or Ga treatment for SCID mice in the experiment shown in Fig. 1B. Mice were infected with 10<sup>6</sup> CFU. Tissues were homogenized, and the numbers of *M. tuberculosis* CFU in the lungs (A), spleens (B), and liver (C) were determined at the time of death of the animals or when they were sacrificed after infection, as described in Materials and Methods. Asterisks indicate a *P* value of <0.05.

100  $\mu$ M, with a maximal inhibition of about 50% being achieved (Fig. 4).

**Gallium inhibits *Mycobacterium tuberculosis* aconitase activity.** Aconitase is an iron-sulfur enzyme that functions as an electron carrier and catalyzes the isomerization of citrate to isocitrate via *cis*-aconitate in the tricarboxylic acid (TCA) cycle; thus, it is involved in ATP production (26). In addition, it has been shown that *M. tuberculosis* aconitase binds to iron-responsive element (IRE)-like sequences within the *M. tuberculosis* genome, indicating a possible role in regulating gene expression at a posttranscrip-

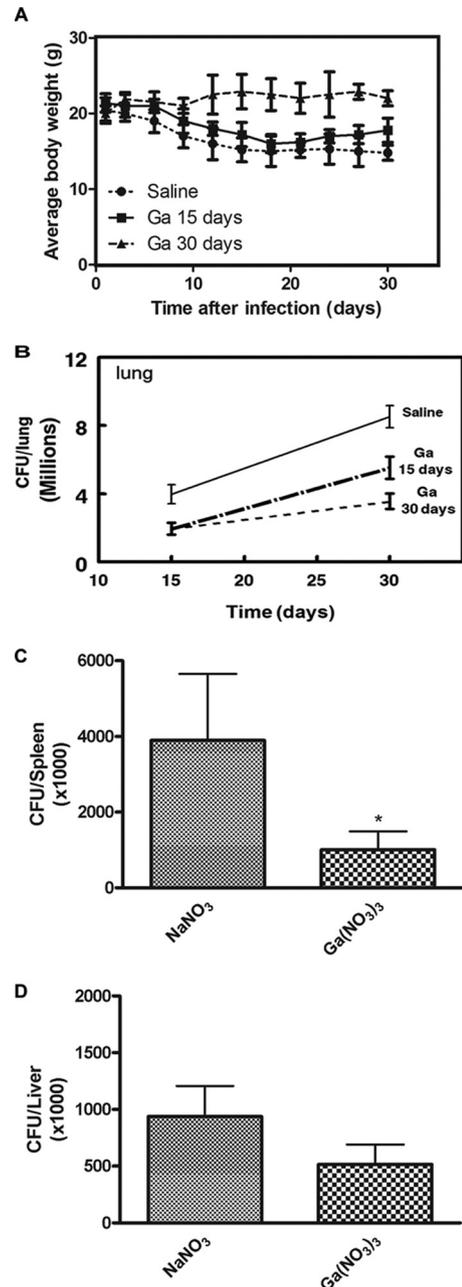


FIG 3 Gallium prevents weight loss and decreases *M. tuberculosis* growth in lungs and other organs of *M. tuberculosis*-infected BALB/c mice. BALB/c mice were infected with 100 CFU of the virulent *M. tuberculosis* Erdman strain i.t., followed by daily intraperitoneal administration of Ga(NO<sub>3</sub>)<sub>3</sub> (10 mg/kg), which was initiated immediately following infection (*n* = 5 each group). For some mice, Ga therapy was stopped after 15 days. Control mice received either NaNO<sub>3</sub> or saline. (A) *M. tuberculosis* infection led to a chronic nonlethal infection that resulted in a loss of weight of the animals over time. Animals that received Ga for either for the full 30 days or the initial 15 days before sacrifice exhibited less weight loss than mice receiving saline. (B) When *M. tuberculosis* CFU in the lungs were determined at 30 days postinfection, mice receiving Ga for the full 30 days showed a significant reduction of CFU (*P* < 0.05) relative to the saline-treated control. When Ga was stopped after 15 days, a significant increase in CFU was still observed in mouse lungs relative to the control (*P* < 0.05). (C) A significant reduction in *M. tuberculosis* CFU was also seen in the spleens of mice treated with Ga for 30 days (*P* < 0.05) compared to NaNO<sub>3</sub> controls. In liver, there was a reduction, but it did not reach statistical significance (*P* > 0.05). Asterisks indicate a *P* value of <0.05.

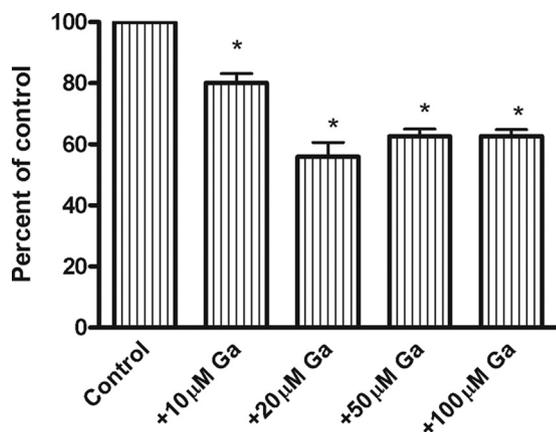


FIG 4 Gallium inhibits *M. tuberculosis* RR activity. RR activity in *M. tuberculosis* was measured as described in Materials and Methods. *M. tuberculosis* H<sub>37</sub>R<sub>a</sub> bacteria were treated with different concentrations of Ga for 24 h. Inhibition of RR was seen at all levels of Ga treatment relative to the control ( $P < 0.05$ ). The inhibition increased significantly when the Ga concentration was doubled from 10 to 20 μM. However, further inhibition was not seen when the Ga treatment dose was increased to 50 or 100 μM. Asterisks indicate a  $P$  value of  $< 0.05$  ( $n = 5$ ).

tional level (27). Therefore, we explored the effect of Ga treatment on the activity of aconitase in *M. tuberculosis*. *M. tuberculosis* was incubated in defined 7H9 medium alone or with the desired concentrations of Ga(NO<sub>3</sub>)<sub>3</sub>, following which the bacteria were harvested and aconitase activity was determined. A Ga-mediated concentration-dependent inhibition of *M. tuberculosis* aconitase activity was observed (Fig. 5).

## DISCUSSION

Drug-resistant tuberculosis has become a major therapeutic challenge (28), and the search for an alternative effective treatment is an urgent necessity. Iron is critical for the growth/metabolism of most bacteria (29), including *M. tuberculosis* (30), making iron metabolism an ideal target for novel therapy. Gallium is a transition metal that has been developed as a therapeutic entity for cancer because it disrupts iron metabolism of eukaryotic cells. Ga can interfere with RR activity and inhibit DNA synthesis (31). It was also shown previously that Ga(NO<sub>3</sub>)<sub>3</sub> can inhibit protein tyrosine phosphatase (PTPase) in Jurkat human T-cell leukemia cells and HT-29 human colon cancer cells (32). In spite of these biochemical effects, citrate-buffered Ga(NO<sub>3</sub>)<sub>3</sub> is an FDA-approved therapy for the treatment of hypercalcemia of malignancy and has been well tolerated (3). The primary toxicity observed has been precipitation of the drug in the kidney following rapid infusion, leading to renal failure. Thus, Ga is a well-established medicinal metal with an established safety profile.

We have previously shown that Ga inhibits the *in vitro* growth of *M. tuberculosis* and *Mycobacterium avium* complex bacteria growing extracellularly or in human macrophages (6). We now extended the previous work by examining the efficacy of Ga in murine models of pulmonary *M. tuberculosis* infection. To provide a highly discriminating and rapid assay to determine the impact of Ga on the course of *M. tuberculosis* infection in mice, we used BALB/c SCID mice in our initial set of experiments. BALB/c SCID mice lack functional B and T cells but exhibit normal function of myeloid cells, antigen-presenting cells, and natural killer

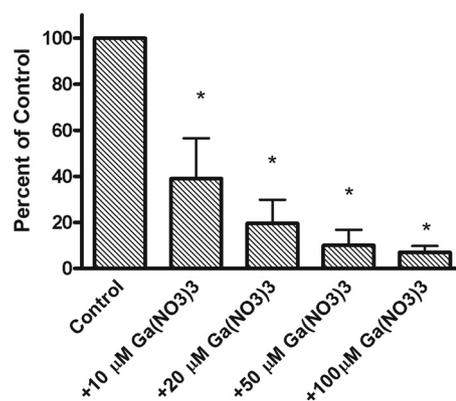


FIG 5 Gallium inhibits *M. tuberculosis* aconitase activity. Aconitase activity in *M. tuberculosis* was measured as described in Materials and Methods. *M. tuberculosis* bacteria were incubated in defined 7H9 medium that was supplemented with 1 μM ferric ammonium citrate alone or with the desired concentrations of Ga(NO<sub>3</sub>)<sub>3</sub> for 24 h, following which the organisms were harvested and aconitase activity was determined. Ga-mediated concentration-dependent inhibition of *M. tuberculosis* aconitase activity was observed. Asterisks indicate a  $P$  value of  $< 0.05$  ( $n = 4$  to 7 for each group).

(NK) cells (33). SCID mice have been used previously to study chronic infections and infections caused by intracellular pathogens, including *M. tuberculosis* (34, 35). The lack of functional B and T cells in SCID mice makes them highly susceptible to infections caused by intracellular pathogens, including *M. tuberculosis* (Fig. 1). As expected, control mice infected i.t. with *M. tuberculosis* succumbed to infection over a matter of weeks in proportion to the initial inoculum of *M. tuberculosis*. Daily administration of Ga i.p. markedly extended the life span of the *M. tuberculosis*-infected animals compared to the controls, regardless of whether a low- or high-dose inoculum of *M. tuberculosis* was used.

Pulmonary *M. tuberculosis* infection of mice leads to rapid dissemination of the organism to other organs (Fig. 2). Ga administration significantly decreased the level of *M. tuberculosis* in the lungs (89.9%), liver (97.7%), and spleen (84.8%) in SCID mice. This magnitude of decrease of *M. tuberculosis* CFU with Ga was less than the profound effect on survival. One interpretation of these data is that in addition to its direct antimicrobial activity, Ga may enhance the effectiveness of the host immune system to kill the organism. In immunocompromised animals, the latter effect of Ga may be absent.

In our second set of experiments, the effect of Ga on *M. tuberculosis* infection in standard BALB/c mice was examined. As expected from the literature (36), i.t. infection of BALB/c mice led to a chronic nonlethal infection that also resulted in a loss of weight of the infected animals over time (Fig. 3). Animals treated with Ga for 30 days exhibited less weight loss, and the number of viable *M. tuberculosis* CFU in lung and other tissues was significantly decreased (Fig. 3). The magnitude of the CFU difference is less than that reported for other standard antibiotics, such as rifampin and isoniazid (37). Our *in vivo* results are also somewhat contrary to our previous work, which showed that Ga treatment led to a several-log decline in CFU in human MDM *in vitro* relative to untreated controls (30). Clearly, more work is needed to optimize the dosing regimen of Ga in this murine model before valid comparisons with other agents can be made. Our laboratory and others have shown the ability of Ga(NO<sub>3</sub>)<sub>3</sub> to inhibit the growth of my-

cobacteria and other bacteria (6, 38). It is possible that the bacteriostatic effect of Ga on mycobacteria is related in part to the inactivation of bacterial Fe-centered enzymes such as aconitase and RR.

The efficacy of Ga in murine TB is consistent with results reported previously for other types of bacterial infections. We have previously shown that Ga is protective in murine lung infection models with both *Pseudomonas aeruginosa* (16) and *Francisella tularensis* (17). Others have reported various forms of Ga to be effective in a murine *P. aeruginosa* burn infection model (39) and in infections caused by *Rhodococcus equi* (40) and *Acinetobacter baumannii* (5).

The animal studies reported here provide an *in vivo* proof of principle that Ga could prove to be effective in the treatment of *M. tuberculosis* infection. Ga's ability to substitute for iron in iron-dependent metabolic pathways represents a fundamental new target approach to treatment. Having said this, further enhancement of the potency of Ga against *M. tuberculosis* requires a better understanding of the specific microbial target(s) of Ga. Our previously reported findings that Ga inhibition of *M. tuberculosis* growth correlates directly with disruption of bacterial iron uptake (30) led us to study the possible effect of Ga on iron-dependent enzymes such as RR, which is essential for DNA synthesis (23). Eukaryotic RR was previously shown to be the key target of the anticancer effect of Ga (23). *M. tuberculosis* contains both class Ib and class II ribonucleotide reductases (24). In the present work, we found concentration-dependent inhibition of *M. tuberculosis* RR activity at Ga concentrations at or below those leading to growth inhibition. However, inhibition plateaued at approximately 50% of RR activity, even at concentrations of Ga that completely inhibited growth (Fig. 4).

These data provide evidence that the ability of Ga to inhibit RR could contribute to the growth-inhibitory effect of Ga on *M. tuberculosis*. Previous work has shown that the class Ib, but not the class II, RR enzyme is essential for growth of *M. tuberculosis* (24). The loss of the class Ib RR enzyme in *M. tuberculosis* is not compensated for by the presence of the type II RR (24). The discrepancy between the relative magnitudes of RR inhibition and growth inhibition by Ga raises the possibility that Ga selectively inhibits the class Ib enzyme of RR and not class II. However, further investigation is needed to identify the specific RR(s) targeted by Ga and the mechanism(s) of inhibition.

Importantly, our previous and current data provide evidence for the specificity of Ga for *M. tuberculosis* RR in the context of TB infection. In this regard, macrophages are terminally differentiated cells and have minimal to no RR activity, and our previous work showing no toxicity of Ga for human macrophages is consistent with this (6). Also, the safety portfolio for Ga is good for the FDA indications (15).

Our data also suggest that in addition to RR, inhibition of another important iron-dependent enzyme could contribute to the ability of Ga to inhibit *M. tuberculosis* growth. *M. tuberculosis* aconitase catalyzes the isomerization of citrate to isocitrate in the TCA cycle and acts as an RNA-binding protein (27). We found that Ga treatment inhibited aconitase in a dose-dependent manner. Thus, this inhibition may be an additional mechanism of Ga action on *M. tuberculosis*. Given the multitude of iron-dependent enzymes in bacteria, it is likely that Ga inhibits several such *M. tuberculosis* enzymes. We have previously shown that growth of *F. tularensis* in the presence of Ga leads to a decrease in the activities

of both catalase and iron superoxide dismutase (17). Additional work is needed to assess which of the multiple potential targets is most critical to the antimicrobial effect of Ga, and this may vary with the bacterial species being studied. For example, preliminary data suggest that Ga has no effect on *F. tularensis* aconitase activity at concentrations that completely inhibit bacterial growth (O. Olakanmi and B. E. Britigan, unpublished data).

In summary, the current study provides further evidence that Ga has promise as an antimycobacterial chemotherapeutic agent that likely acts by interfering with iron uptake and utilization by *M. tuberculosis*. The current work was undertaken using Ga(NO<sub>3</sub>)<sub>3</sub> because it is the FDA-approved formulation of Ga that is approved for use in humans and has an established safety profile. However, alternative, more potent Ga formulations and delivery systems are worth examining. Previous work by Stojiljkovic et al. (18) suggested that Ga-protoporphyrin complexes exhibit potent activity against mycobacteria *in vitro*, an observation that we have recently confirmed (B. L. Switzer, M. Y. Abdalla, and B. E. Britigan, unpublished data). An oral formulation of Ga in the form of Ga-maltolate has been developed and appears to have good absorption from the gastrointestinal tract and bioactivity (3). Ga represents a new class of antibiotics with some specificity and is cheap to produce. Further efforts to identify and develop Ga formulations that optimize the antimicrobial effect while limiting toxicity to the host are needed and are under way in our laboratory and those of other investigators.

## ACKNOWLEDGMENTS

This work is based upon work supported in part by the Department of Veterans Affairs through a merit review grant to B.E.B.

The contents do not necessarily represent the view of the Department of Veterans Affairs or the U.S. Government.

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