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Clofazimine as a comparator for preclinical efficacy evaluations of experimental therapeutics against pulmonary *M. abscessus* infection in mice

Divya Sriram,

Rishi Wahi,

Emily C. Maggioncalda,

Chandra M. Panthi,

Gyanu Lamichhane*

Division of Infectious Diseases, Department of Medicine, School of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21287.

Abstract

Mycobacteroides abscessus (*Mab,* also known as *Mycobacterium abscessus*) can cause chronic pulmonary disease in the setting of structural lung conditions. Current treatment recommendations require at least one year of daily therapy with repurposed antibiotics. Yet these therapies are often ineffective and associated with significant adverse events. To address this challenge, research efforts are underway to develop new antibiotics and regimens. During the preclinical phase of treatment development, experimental agents require testing and comparison alongside positive controls that are known agents with clinical history. As there are no FDA approved treatments for this indication, here, we have considered repurposed antibiotics currently included in the recommendation for treating *Mab* disease as candidates for selection of an ideal standard comparator that can serve as a positive control in preclinical studies. Clofazimine meets the criteria for an ideal positive control as it can be administered via the least invasive route, requires only once-daily dosing, is well tolerated, and is widely available in high purity from independent sources. Using a mouse model of pulmonary *Mab* disease, we assessed for ideal dosages of clofazimine in C3HeB/FeJ and BALB/c mice in a six-week treatment window. Clofazimine, 25 mg/kg, once daily, produced desired reduction in *Mab* burden in the lungs of C3HeB/FeJ and BALB/c. Based on these findings, we conclude that clofazimine meets the criteria for a positive control comparator in mice for use in preclinical efficacy assessments of agents for treatment of

*To whom correspondence should be addressed: Johns Hopkins University School of Medicine, 1550 Orleans St, Room 107, Baltimore, MD 21287. lamichhane@jhu.edu.

AUTHOR CONTRIBUTIONS

DS: analyzed data, prepared figures, and manuscript. RW: analyzed data and prepared manuscript. ECM designed study and prepared final draft of manuscript. CMP: undertook animal studies. GL: conceived and supervised study, analyzed data and prepared final draft of manuscript.

DECLARATION OF COMPETING INTEREST

All authors declare no conflict of interest.

Mab pulmonary disease. Although not included in the current standard-of-care for treating *Mab* disease, rifabutin, 20 mg/kg, also produced desired *Mab* lung burden in C3HeB/FeJ mice but not in BALB/c mice.

Keywords

M. abscessus ; experimental therapeutics; clofazimine; rifabutin; positive control

1. INTRODUCTION

Mycobacteroides abscessus (*Mab*) can cause opportunistic pulmonary infections in patients with chronic lung conditions such as cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease [1,2]. It is also known to cause diseases in the skin and soft tissues and can be fatal if it disseminates [3–7]. *Mab* lung disease is often misdiagnosed as tuberculosis as clinical presentations of *Mab,* and *Mycobacterium tuberculosis* lung diseases bear significant resemblance. *Mab* was recently reclassified into genus *Mycobacteroides* due to its distinct genetic differences from members of the *Mycobacterium* genus [8]. The *Mab* species is further classified into three sub-species- *abscessus*, *massiliense,* and *bolletii* [9]. A higher incidence of subsp. *abscessus* and subsp. *massiliense* was reported in the US and East Asia, while the incidence of infections with subsp. *bolletii* was rare [10]. Variations in the subsp. incidence rates between different geographical regions have also been reported [11]. The incidence of *Mab* infections has been gradually increasing in the US [12–14].

Mab infection, like tuberculosis, is a chronic infection and is treated with a regimen containing a cocktail of antibiotics given in phases that last for at least 12 months [15–17]. Despite the multi-drug therapy that is continually administered for a long duration, the treatment success rate is 30–50%, and the culture conversion rate is between 25% and 88% [11,18–22]. Several factors are considered pertinent to poor outcomes of current treatment regimens. First, the minimum inhibitory concentration (MIC) of most of the antibiotics available today is higher than what is physiologically attainable in human blood, and hence *Mab* is considered to be intrinsically resistant to most antibiotics [16,23–26]. Second, the current recommendations are not based on clinical trials and comprise of repurposed antibiotics that are not approved by the FDA for this indication [16,17]. Third, significant adverse events have been reported for several antibiotics included in the treatment regimens necessitating careful monitoring of patient response and revision of prescriptions [27,28]. Hence, patients with *Mab* infection have limited treatment options. To address this challenge, efforts at developing new antibiotics have begun in earnest.

Naturally, the first steps towards developing new treatments for *Mab* disease have focused on *in vitro* evaluations of activities of new agents and those approved for other indications. These studies have identified several new candidates such as PIPD1, an indole-2-carboxamide [29–32], epetraborole [33,34], oxazolidinones LCB01–0371 [35] and tedizolid [36], and a penem T405 [37–39]. In addition, tetracyclines such as omadacycline and eravacycline [40–45] and a spectrum of β-lactams have been evaluated for their activities against *Mab* [46–50]. β-lactamase inhibitors such as sulbactam, tazobactam,

clavulanate, relebactam, vaborbactam, and avibactam have been evaluated as companions to β-lactams against *Mab* [51–55]. For some of these candidates, preclinical efficacy evaluations in animal models that mimic key features of *Mab* disease in humans have been described [31,33,34,45,47,56,57]. Recently, phage therapy has surfaced as a potential treatment option for multi-drug resistant *Mab* infections and is being tested in clinical settings [58–60]. New combination regimens comprising of repurposed drugs are also being evaluated against *Mab* [32,47,57,61]. It is necessary to compare efficacy of an experimental agent with that of a known standard-of-care drug to assess if desirable clinical activity can be expected from the experimental agent.

The following practical therapeutic attributes are desirable in a positive control comparator antibiotic for use in preclinical efficacy assessment studies [62,63]: (i) **requires low dosing frequency** (once daily or less): To reduce variability in the experiment due to multiple interferences during dosing and to introduce the least amount of stress to animals, once daily or lower frequency of administration would be ideal for preclinical use. A low dosing frequency is also less effort- and resource-intensive and, therefore, logistically desirable. (ii) **produces tolerable adverse events in animal models**: Antibiotics that cause adverse events in animal models make it difficult to delineate the desirable targeted effect of the drug from its undesirable *in vivo* toxicity. The use of such drugs would thus, reduce confidence in the experiment and interfere with the interpretation of the antibiotic efficacy. Therefore, such antibiotics would not be preferred as comparator control antibiotics. (iii) **is reliably available from multiple independent sources:** For the antibiotic to be adopted and used widely and sustainably as a comparator control, it must be reliably available in high purity from independent vendors. This enables independent laboratories to undertake efficacy evaluations and contribute to the development of new therapeutics to treat *Mab* disease. (iv) **can be administered via the least invasive and most convenient route in the intended model animal**: we reviewed the literature for precedent for the types of model animals used in mycobacterial research and for routes of drug administration. Although larger animals such as guinea pigs, rabbits and macaques are also used, mice have been the choice in overwhelming number of studies and have successfully predicted drug and regimen efficacies in humans [64–66].

There is rich literature for preclinical drug development for diseases caused by *Mycobacterium tuberculosis*, an organism related to *Mab*. In these studies, standard-of-care antibiotics like isoniazid, rifampin, and pyrazinamide have been effectively used as positive control comparators against experimental agents [67–71]. The use of such controls has enabled harmonizing of protocols across laboratories, which has allowed for a more reliable comparison of findings. These antibiotics are administered orally. While intravenous route bypasses the need for adsorption, and there are several anti-tuberculosis drugs that are administered intravenously in humans, such as amikacin, capreomycin, kanamycin and meropenem, intravenous administration in mice is extremely labor intensive as locating and reliably injecting via the tail vein in large numbers of mice, each day for several weeks is logistically impractical and undesirable. Other routes of injection such as subpleural and subcutaneous can be undertaken reliably, but daily injections over several weeks often leads to puncture wounds and ulcers due to repeated trauma at the sites of injection. Perhaps

because of these reasons, oral routes have been used in the overwhelming majority of efficacy evaluations against mycobacterial diseases.

We have observed the disadvantages of long-term injections through our previous studies on a model of pulmonary *Mab* disease using the C3HeB/FeJ mouse strain [72]. In some of these studies, imipenem, a parenteral drug, was used and had to be administered via subcutaneous injections in mice, twice-daily for four-weeks [39,45]. While feasible, injecting large numbers of mice daily for several weeks was far more resource and time intensive compared to similar treatments delivered via oral gavage in mice. For instance, in evaluation of efficacy of an experimental agent T405 [39], 196 needle-syringe combinations (single-use) were required. In comparison, only one oral gavage needle would be sufficient for the same study if the drug could be administered orally as the gavage needle can be rinsed and sterilized in 70% ethanol after each use. We also observed fibrosis of skin tissue at injection sites, necessitating administration via other sites. Oral route of administration (voluntary consumption, orogastric or nasogastric gavage) overcomes these challenges, is less invasive and, less resource- and time-intensive.

Various antibiotics are repurposed as standard-of-care for *Mab* disease, including amikacin, azithromycin, bedaquiline, cefoxitin, ciprofloxacin, clarithromycin, clofazimine, doxycycline, ethambutol, imipenem, linezolid, minocycline, moxifloxacin, omadacycline, tigecycline, and trimethoprim [16,17]. Prior preclinical efficacy studies have used different comparator antibiotics in different dosages such as amikacin 150 mg/kg [31,57], or 250 mg/kg [33], clarithromycin 200 mg/kg [35], imipenem 200 mg/kg administered twice daily [39,45], linezolid 100 mg/kg, and rifabutin (10 mg/kg) [34]. Amikacin and imipenem are both injectable, and therefore, they also are less convenient than oral drugs for administration in mice. Clarithromycin and linezolid are used in very high doses and given twice daily, hence do not meet the criteria. The use of different antibiotics has made it challenging to harmonize protocols and compare efficacies of experimental agents against *Mab* across preclinical studies. Therefore, there is a need to identify a drug with ideal features of a positive control comparator that can be included in preclinical efficacy evaluations of experimental agents against *Mab*.

The aim of this study is to identify one standard-of-care drug that meets the above-mentioned criteria and can serve as a positive control comparator in efficacy studies against *Mab* disease. We considered all antibiotics included in the current standard-of-care for *Mab* disease treatment and assessed them against the above-mentioned criteria. Clofazimine and moxifloxacin meet all criteria. We evaluated the efficacy of clofazimine against *Mab* infections in two mouse strains that have been extensively used in preclinical studies of mycobacterial infections, including *Mab*: C3HeB/FeJ [47,57,72–75] and BALB/c [69,76]. Based on the findings, we describe clofazimine dose and dosing frequency for preclinical efficacy testing of experimental agents against *Mab* disease. This satisfied the overall aim of this study to identify one standard-of-care drug with ideal features to serve as a positive control comparator. Although rifabutin is not included in the current recommendations to treat *Mab* disease, there is an increasing interest in repurposing this drug to treat *Mab* disease [77]. Therefore, we also evaluated rifabutin against *Mab* pulmonary infections in C3HeB/FeJ and BALB/c mice to assess for attributes of an ideal positive control comparator.

2. MATERIALS AND METHODS

2.1 Bacterial strains and *in vitro* growth conditions.

M. abscessus strain ATCC 19977, which has been historically considered a reference *Mab* strain, was used [78]. This strain was procured from ATCC (Manassas, VA) and authenticated by genome sequencing [48]. Middlebrook 7H9 broth (catalog no. 271310, Difco) supplemented with 0.5% glycerol, 10% albumin-dextrose-salt enrichment and 0.05% Tween-80 was used to culture *Mab* as described [79]. *Mab* cultures were grown in an orbital shaker at 220 RPM, 37 °C. Middlebrook 7H11 selective agar (catalog no. 283810, Difco), supplemented with 0.5% glycerol, 10% albumin-dextrose-salt enrichment, 0.05% Tween-80, 50 μg/ml carbenicillin (catalog no. C46000, Research Products International) and 50 μg/ml cycloheximide (catalog no. C7698, Sigma-Aldrich) was used to recover *Mab* from mouse lung homogenates. When grown on Middlebrook 7H11 agar, both rough and smooth colonies of *Mab* ATCC 19977 were observed.

2.2 Efficacy determination in mice.

C3HeB/FeJ mice (Jackson Laboratories, Bar Harbor, ME) and BALB/c mice (Charles River Laboratories), 5–6 weeks old, female, were used as described in the protocol for the mouse model of pulmonary *Mab* infection used in this study [72] and in studies in which imipenem was used as a positive control comparator [45,47]. Only female mice were used as in our experience male mice exhibit aggressive behavior including biting the handler. As the study requires daily administration of drugs in large numbers of mice, inclusion of only females allowed us to exclude avoidable challenges and undertake the studies in a safe manner. Beginning a week prior to infection and continuing throughout the study, 5 mg/kg/day dexamethasone was administered to each mouse as specified in the publication that described this mouse model of pulmonary *Mab* infection [37]. Dexamethasone (catalog no. D1756, Sigma-Aldrich) was dissolved in sterile 1x phosphate buffered saline, pH 7.4 (catalog no. 114-058-101CS, Quality Biologicals) to a concentration of 1.25 mg/ml and 0.1 ml bolus was administered once daily, seven days a week, by subcutaneous injection into the hind dorsal tissue using 27-gauge needle (catalog no. 305620, Beckton and Dickinson). *Mab* was grown to exponential phase in Middlebrook 7H9 broth and was used to prepare a 10 ml infecting suspension at an optical density, A_{600nm} = 0.1. For the pilot study in which treatments were administered for three weeks, C3HeB/FeJ (n=60) were infected simultaneously with aerosolized suspension of *Mab* in an inhalation chamber according to manufacturer's guidelines (Glas-Col, Terre Haute, IN). The infection cycle included preheating for 15 min, aerosol nebulization for 30 min, and cloud decay for 30 min, followed by surface decontamination for 15 min. Separately BALB/c (n=60) mice were infected using the identical protocol. Five mice were allocated for determination of *Mab* implantation following infection and five additional mice were allocated for determination of *Mab* burden at one week following infection, the time at which antibiotic treatment was initiated. One week following infection, mice were randomly allocated into five groups of 10 mice per group. Treatment was administered once daily, and 0.2 ml bolus of each agent was administered via oral gavage. Mice in the first group were administered 1x PBS, pH 7.4, as this buffer was used as the solvent for clofazimine and rifabutin, and therefore represent the negative control group. Mice in the second and third groups received 25 and 50 mg/kg

clofazimine, respectively. Similarly, mice in the fourth and fifth groups received 10 and 20 mg/kg rifabutin. *Mab* burden in the lungs of mice were determined at 24 hours post-infection (designated week −1), one week following infection (week 0), and at one- and three weeks following treatment initiation (designated week +1 and +3, respectively).

Similarly, in the subsequent study in which treatments were administered for six weeks, C3HeB/FeJ (n=55) and BALB/c (n=55) were infected simultaneously with aerosol of *Mab*. Five mice were allocated for determination of *Mab* implantation following infection and five additional mice were allocated for determination of *Mab* burden at one week following infection. At this time, the remaining 45 mice were randomly allocated into three groups of 15 mice per group and antibiotic treatment was initiated. 1xPBS, pH 7.4, was administered to the first group, 25 mg/kg clofazimine was administered to the second group and 20 mg/kg rifabutin was administered to the third group.

Clofazimine was procured from Sigma-Aldrich (catalog no. C8895) and rifabutin was procured from Octagon Chemicals Limited (CAS# 72559-06-9). Suspensions of these drugs were prepared in 0.05% agarose as described [80]. To prepare 0.05% agarose, 500 mg Bacto Agar (catalog no. 214010, Becton Dickinson) was added to 1000 ml 1x PBS, pH 7.4, autoclaved for 10 minutes and cooled to room temperature. To deliver 25- and 50 mg/kg doses of clofazimine, 3.125- and 6.25 mg/ml suspensions were prepared, respectively, and 0.2 ml was administered to each mouse via oral gavage, once daily, seven days a week. Similarly, 1.25- and 2.5 mg/ml rifabutin suspensions prepared in 0.05% agarose were used to deliver 10 and 20 mg/kg rifabutin, respectively.

Five mice were sacrificed per time point per group, lungs were obtained and homogenized in a tube containing 900 μl 1x PBS, pH 7.4, and 0.2 mm glass beads in a mechanical homogenizer (Minilys, Bertin Technologies) at 5,000 RPM for 0.5 min. 100 μl of undiluted lung homogenates and 10-fold dilutions prepared in 1x PBS, pH 7.4 were inoculated onto Middlebrook 7H11 selective agar, incubated at 37 °C for 5 days and CFU was enumerated. . CFU counts from each mouse lung were converted into CFU per lung, comprising the average of three consecutive steps of a 10-fold dilution series of a given lung sample. Mean CFU ± standard error of the mean (SEM) in five mice per group per time point was plotted to determine the determine *Mab* burden in the lungs of mice.

2.3 Data and Statistics:

Mab CFU burden in the lungs of each mouse (n=5 per group, per time-point) was determined as described above. Mean *Mab* lung CFUs data was graphed as a function of time ± SEM. Statistical comparisons of CFUs at end time-point between different PBS vs treatment groups were performed by unpaired one-tailed *t*-test. Significance was determined at 95% confidence intervals ($p<0.05$ was considered significant). One-tailed t-tests were used to determine whether the performance of treated group was higher than the untreated control group (PBS), thus indicating the efficacy of the tested drugs (clofazimine or rifabutin).

2.4 Ethics.

Animal procedures used in the studies described here were performed in adherence to the Johns Hopkins University Animal Care and Use Committee and to the national guidelines.

3. RESULTS

3.1 Criterial for consideration as a positive control comparator:

The antibiotics used in the current regimens to treat *Mab* disease and whether they meet each of the above-mentioned criteria for an ideal positive control comparator are listed in Table 1 [16,17]. As clofazimine met all criteria and is one of the first-line antibiotics recommended to treat *Mab* disease, it was selected as a candidate for dose-efficacy evaluations in the pulmonary *Mab* infection models in C3HeB/FeJ and BALB/c mice [72]. Prior studies have reported once-daily 20 mg/kg clofazimine to be effective at reducing *Mab* burden in the lungs of mice [56,81]. A dose range of 25–100 mg/kg was demonstrated to be efficacious against *M. tuberculosis* infection in mice [82]. Independent studies have reported clofazimine MIC of 0.25–4.0 μg/ml against *Mab* isolates [25,83–87]. Current guidelines for treatment of *Mab* lung disease recommend once daily, 1.5 mg/kg, clofazimine in humans [88]. Using validated methods to determine animal equivalent dose (AED) [89], we determined that for clofazimine, a dose of ~19 mg/kg, once daily, is equivalent in C3HeB/FeJ and BALB/c mice. According to this method, AED = Human dose (mg/kg) x Km ratio (for mouse Km ratio is 12.3). Based on these prior findings, we selected two dosages of clofazimine for further evaluation: a lower dose of 25 mg/kg and a higher dose of 50 mg/kg. Evaluations of clofazimine in C3HeB/FeJ and BALB/c mice was undertaken in two phases. In the initial phase, the pilot study, we limited the duration of efficacy evaluation to three weeks of antibiotic treatment as the goal was to identify the lower dose of clofazimine, between 25- and 50 mg/kg, which would produce a reduction in *Mab* burden in the lungs of these mice.

3.2: Clofazimine, 25- and 50 mg/kg; treatment duration three weeks:

The mean *Mab* lung burdens at implantation (week −1) were 2.82 and 3.39 $\log_{10}$ CFU in C3HeB/FeJ and BALB/c mice, respectively (Figure 1A and 1B). One week following infection, when antibiotic treatment was initiated (week 0), the mean *Mab* lung burdens were 3.99 and 4.66 $\log_{10}$ CFU in C3HeB/FeJ and BALB/c mice, respectively. At this time, mice were randomly allocated to antibiotic treatment groups, with ten mice per group, and once-daily antibiotic treatment was initiated. Mice in the negative control group were administered PBS as it was used as the vehicle to prepare clofazimine formulations. Mice in the clofazimine treated group were administered either 25 or 50 mg/kg clofazimine. *Mab* lung burden steadily increased throughout the duration of the study in both C3HeB/FeJ and BALB/c mice treated with PBS, thereby reproducing prior observations [45,47,72].

In C3HeB/FeJ mice treated with 25-or 50 mg/kg clofazimine, *Mab* lung burden failed to decline during the three-week treatment duration of this study (Figure 1A). At the three-week time point, the mean *Mab* lung burden of C3HeB/FeJ mice treated with 25- or 50 mg/kg clofazimine was statistically insignificant compared to that of PBS-treated mice as determined by one-tailed *t*-test with p=0.3973 and p=0.2444, respectively. In BALB/c

mice treated with 25 mg/kg clofazimine, *Mab* lung burden remained steady at the end of first week but was ~1.64 $\log_{10}$ lower at the end of the third week of treatment. Whereas 50 mg/kg clofazimine produced a consistent reduction in lung *Mab* CFU throughout the treatment period (Figure 1B). At the end of third week, the mean *Mab* lung CFU in BALB/c mice treated with 25 mg/kg or 50mg/kg clofazimine group was statistically significant compared to the PBS group, as determined by one-tailed *t*-test with p=0.0159 and p= 0.0317, respectively.

Based on the *Mab* lung CFU reduction during the later stage of treatment in BALB/c mice treated with 25 mg/kg of clofazimine (Figure 1B), and prior reports of delayed bactericidal response in mice infected with *M. tuberculosis* [82], we hypothesized that the lower dose of clofazimine considered here, 25 mg/kg, might be sufficient to exhibit efficacy if administered for a longer treatment duration. Therefore, we considered 25 mg/kg clofazimine for further evaluation.

The existing recommendations for treating *Mab* lung disease require administration of antibiotics for one year or more [16,17]. For efficacy evaluations of experimental agents in mice to be informative for studies in humans, it is likely that *Mab* burden in the lungs of mice will need to be evaluated for an extended duration. Therefore, in the second phase of the study, clofazimine administration duration was extended to six week and the efficacy of once daily 25 mg/kg oral treatment was evaluated in both C3HeB/FeJ and BALB/c mice.

3.2 Clofazimine, 25 mg/kg; treatment duration six weeks:

In this study, the mean *Mab* lung burdens at implantation (week −1) were 3.21 and 3.15 $\log_{10}$ CFU in C3HeB/FeJ and BALB/c mice, respectively (Figure 2). One week following infection, when antibiotic treatment was initiated (week 0), the mean *Mab* lung burdens were 3.75 and 3.80 $\log_{10}$ CFU in C3HeB/FeJ and BALB/c mice, respectively. In BALB/c mice, compared to that in three-week study (Figure 1b), the initial inoculum was slightly lower and so was the CFU at the initiation of treatment. Although exact protocol was used for infection, this level of variation can be expected in biological repeats. At this time, mice were randomly allocated to different treatment groups, with 15 mice per group, and once-daily treatment was initiated. As in the first study, mice that were administered PBS represent the negative control group. To mice in the test group, 25 mg/kg clofazimine was administered once daily. *Mab* lung burden steadily increased throughout the duration of the study in both C3HeB/FeJ and BALB/c mice that received PBS (Figure 2A and 2B), thereby reproducing prior observations [45,47,72].

In C3HeB/FeJ mice treated with 25 mg/kg clofazimine, there was a slight increase in mean lung *Mab* burden at two weeks followed by 1.41 $\log_{10}$ (compared to week 0) and an additional 1 $\log_{10}$ decline by four- and six-week time-points, respectively. Overall, 25 mg/kg clofazimine produced 2.42 $\log_{10}$ reduction in lung *Mab* burden in C3HeB/FeJ mice over the six-week treatment period (Figure 2A). In BALB/c mice, 25 mg/kg clofazimine produced 3.22 $\log_{10}$ reduction in *Mab* burden during the period and therefore exhibited activity similar to that in C3HeB/FeJ mice (Figure 2B). At six weeks, the average *Mab* lung CFU in mice treated with 25 mg/kg clofazimine in both mice strains was statistically significant compared

to PBS group, as determined by one-tailed *t*-test with p= 0.0130 for C3HeB/FeJ mice and p= 0.0014 for BALB/c mice.

Apart from clofazimine, we also investigated, rifabutin, one of second-line antibiotics that met all four criteria of an ideal positive comparator (Table 1). A recent study demonstrated that 10 mg/kg rifabutin, administered once daily, reduces *Mab* burden in the lungs of mice [91]. Studies evaluating rifabutin efficacy against *Mycobacterium avium* have reported the effective dose range to be 5–40 mg/kg, once daily [92–94]. Independent publications have reported MIC of rifabutin of 0.25–32 μg/ml against *Mab* [61,91,95]. Based on the prior efficacy finding against *Mab* and *M. avium* infections and the wide range of MIC, we selected 10 mg/kg as the lower and 20 mg/kg as the higher dose of rifabutin for evaluation in C3HeB/FeJ and BALB/c mice infected with *Mab*. Similar to the clofazimine study, efficacies of these two doses were evaluated with a treatment duration of three weeks. One dose was selected from this study and subsequently evaluated in both mouse strains over a treatmen duration of six weeks.

3.3 Rifabutin, 10mg/kg and 20 mg/kg; treatment duration three weeks:

Mab lung burden failed to decrease in C3HeB/FeJ or BALB/c mice treated with 10 mg/kg rifabutin (Figure 3A & 3B). In C3HeB/FeJ mice, while the mean lung burden in 20 mg/kg rifabutin treated group was statistically insignificant at three-week time-point as determined by one-tailed *t*-test; p=0.0520, this was barely below the 95% confidence interval. Overall, a reduction of 1.58 $\log_{10}$ CFU occurred in the lungs of these mice at the culmination of three weeks of treatment (Figure 3A). In BALB/c mice treated with 20 mg/kg rifabutin, while lung *Mab* CFU increased at the end of the first week of treatment, in the following two weeks, there was a 1.83 $\log_{10}$ reduction in lung *Mab* CFU (Figure 3B). Mean *Mab* lung CFU of this group at the final time-point was statistically significant compared to PBS (one-tailed *t*-test, p=0.0196). To assess whether rifabutin showed delayed bactericidal effect like clofazimine in this study in C3HeB/FeJ mice and efficacy in BALB/c mice, 20 mg/kg dose was selected for assessment for a six-week treatment duration.

3.4 Rifabutin, 20 mg/kg; treatment duration six weeks:

In C3HeB/FeJ mice, lung *Mab* burden remained unchanged at the end of the two-week time-point (Figure 4A). At the four- and six-week time-points, lung *Mab* burden was reduced by 0.84 $\log_{10}$ and an additional 1.16 $\log_{10}$ CFU, respectively. Overall, 20 mg/kg rifabutin produced 2 $\log_{10}$ reduction in lung *Mab* burden in C3HeB/FeJ mice over the six-week treatment period. At the six-week time-point, *Mab* lung burden in C3HeB/FeJ mice treated with 20 mg/kg rifabutin was statistically significant compared to the control group that received PBS treatment (one-tailed *t*-test; p=0.0196). However, in BALB/c mice, 20 mg/kg rifabutin did not produce reduction in lung *Mab* burden during the study period (Figure 4B). Instead, a steady increase in *Mab* lung burden was observed at four- and six-week time-points.

4. DISCUSSION

Developing a new drug is a long and expensive process as most candidates are rejected at various stages of development for not meeting criteria such as safety or efficacy. Prior to assessing safety and efficacy in a clinical trial, similar assessments are made in the preclinical and controlled laboratory setting [96]. In preclinical studies, the inclusion of a drug used as standard-of-care allows for direct comparison of activity of the experimental agent against the known activities of a drug with clinical experience. A standard-of-care drug serves as a benchmark for expected efficacy and safety and, therefore is a positive control comparator for a study. In addition, it serves as an instrument to measure reproducibility among different studies. This aspect is important as the same experimental agent is often evaluated by independent groups using protocols with multiple variables, such as different models of a disease or independent experimental setups. Inclusion of the same positive control comparator(s) as a constant across independent studies permits assessment of the reproducibility of studies, meaningful comparisons of studies, and subsequently combining findings and generating conclusions with higher statistical power. Therefore, the availability of robust positive control comparator(s) is vital to facilitate independent preclinical assessments of an experimental agent.

Preclinical efficacy evaluations of drugs against mycobacterial diseases require assessment over a long duration to mimic treatment durations of several months in humans. As mycobacterial burden needs to be assessed at multiple time-points over an extended duration, a large number of animals are required to complete these evaluations. The experimental drug, and the comparator need to be administered frequently (at least a few times a week, or daily or multiple times a day). For these requirements, the oral route is less invasive for mice and also more convenient for the personnel administering these treatments. Administration via the oral route also requires less resources as the gavage needle is cleaned and reused while the injection needle needs to be discarded after each use. There are numerous reports describing the preference of oral administration in humans with focus on benefits of switching from parenteral to oral treatment regimens [97–100]. This has been supported by several campaigns and initiatives as well, by regulatory bodies like American Board of Internal Medicine Foundation. Their “Choosing Wisely” campaign recommends preference be given to oral formulations of antimicrobials, subject to their high bioavailability [101]. In fact, most of these switches have been possible for oral antibiotics that achieve required bioavailability similar to parenteral and have thus been deemed effective for continued use. Some other advantages of oral antibiotics are: avoiding risk of cannula-related infections or thrombophlebitis, in addition to outright benefits of lower cost of drugs and treatment as it obviates the requirement of a health professional or equipment to administer intravenous antibiotics [102]. Oral antibiotic therapy also potentiates early discharge from the hospital or prevent hospitalization from emergency rooms [103].

Preclinical efforts to develop drugs and regimens to treat *Mab* disease are underway. As there are no FDA-approved drugs to treat this indication, there are no obvious candidates to be included in these studies as positive controls. We identified clofazimine as a candidate for positive control comparator as it is among the standard-of-care drugs for treating *Mab* disease and it met the criteria expected in an ideal positive control. Although clofazimine

is not approved by the FDA for this indication, there is an increasing experience of its repurposing for treating *Mab* disease [16,104]. Additionally, there is a significant clinical experience with clofazimine to treat other mycobacterial diseases [105–107]. Among the antibiotics recommended for *Mab* treatment, moxifloxacin also met all four criteria for ideal positive comparators. As the main aim of our study was to identify one standard-of-care drug to treat *Mab* disease, and clofazimine exhibited the desired attributes of a positive control comparator in two strains of mice, it was beyond the scope of this study to consider additional standard-of-care drugs that met the initial screening criteria, such as moxifloxacin. Although we did not include cost of unit of a drug as a major criterion, clofazimine is currently available at $52 per gram (Sigma-Aldrich, catalog# C8995), whereas moxifloxacin is listed at $1000 per gram (Sigma-Aldrich, catalog# SML1581). As positive control comparators need to be included repeatedly, an agent that is significantly more affordable can be argued to be more accessible.

Azithromycin and rifampicin satisfied three out of four criteria for an ideal positive comparator antibiotic (Table 1). Another reason to exclude azithromycin was that two subspecies of *Mab*, subspecies *abscessus* and *bolletii*, are known to exhibit inducible resistance to macrolides [108]. We restricted our study to clofazimine as it is among the front-line drugs used in standard-of-care for *Mab* disease treatment today. Based on these precedents, studies were designed to identify the dose of clofazimine that would produce a reduction in *Mab* burden in the lungs of mice. Two different strains of mice, C3HeB/FeJ and BALB/c, were considered as they are often used in preclinical efforts to develop new drugs and regimens to treat *Mab* disease.

In summary, clofazimine exhibited bactericidal activity from the third and fourth weeks of treatment not only in C3Heb/FeJ (Figure 2A) but also in BALB/c mice (Figure 2B), indicating delayed bactericidal activity. Clofazimine is known to exhibit delayed *in vivo* bactericidal activity against *M. tuberculosis*, with reduction in lung burden observable only after two weeks of treatment [82,109]. In the pilot study of clofazimine, *Mab* lung burden remained steady at the end of three-week time-point in C3HeB/FeJ mice (Figure 1A). In the subsequent study, *Mab* lung burden in C3HeB/FeJ mice decreased at week-four and -six time-points (Figure 2A). It is likely that reduction in *Mab* burden began after three weeks of clofazimine treatment as delayed antimycobacterial activity has been reported for clofazimine. We have considered clofazimine carry-over from lung homogenates onto agar plates and assessed whether the carry-over occurs in sufficient concentration to affect growth of *Mab* and produce CFU that is lower than in the lungs. There are two aspects to clofazimine carry-over during the experiment. First is whether clofazimine accumulates in the lungs to a level greater than its MIC vs *Mab*. This would produce fewer *Mab* CFUs on agar plates than in the lungs. Clofazimine is known to accumulate in macrophages and fatty tissue in mice, but its concentration in serum of C3HeB/FeJ and BALB/c mice only increases from 1 μg/ml to <2 μg/ml from 4 to 12 weeks of treatment [80]. the MIC of clofazimine vs. *Mab* is 0.25–4.0 μg/ml. The second aspect to this is whether the concentration of clofazimine in the lung homogenates that are inoculated onto agar plates to recover *Mab* is >MIC to affect *Mab* growth and recovery. As each mouse lung was homogenized in nine-fold excess PBS, clofazimine becomes 10-fold diluted in the homogenate. This homogenate is further diluted 10-fold serially prior to inoculating on agar

plates. As only 100 μl of pure or diluted lung homogenate is inoculated onto 25 ml agar, the effective concentration of clofazimine on the agar is diluted at least 100-fold compared to that in the lungs. Therefore, the final concentrations of clofazimine on agar plates is several folds below its MIC to affect *Mab* growth. Agar containing activated charcoal have been used to absorb excess drugs [80], but this was deemed unnecessary in our study as 25 mg/kg clofazimine treatment in C3HeB/FeJ mice resulted in slight increase in *Mab* CFU burden in the initial pilot study (Figure 1), which provided evidence that clofazimine tissue accumulation and carryover from lung homogenates does not reduce *Mab* CFU on agar plates. At the time of this publication, two clinical trials aimed at evaluating efficacy of clofazimine to treat *Mab* disease in humans were underway. Efficacy of regimen containing clofazimine to treat *Mab* disease, but not as a single agent, is being evaluated in a clinical trial NCT04310930 [110]. Another study, NCT05294146 [111], is aimed at optimizing the dose of clofazimine to treat nontuberculous mycobacteria infections, including but not limited to *Mab*. Findings from these and relevant future studies will provide insight into the role of clofazimine in treatment of *Mab* disease in humans.

The delayed activity of clofazimine versus *Mab* observed herein makes them suitable for long-term efficacy studies but limits their usage in short-term studies. Since the duration of *Mab* disease treatment in humans often extends beyond 12 months, preclinical studies with extended treatment durations are likely to be more informative for treatment outcomes in humans. Similar to clofazimine, 20 mg/kg dose of rifabutin also showed a delayed bactericidal activity in C3Heb/FeJ mice, indicating its use in long-term studies in this mice strain (Figure 3A and 4A). However, it is evident from literature that short-term studies are performed especially for proof-of-concept or when new candidate agents are available in quantities that are sufficient only for limited study durations, as is often the case for agents that are difficult to scale in academic settings. In such instances, a positive control comparator that produces reduction in *Mab* burden immediately following treatment is necessary. Prior studies have demonstrated that administration of imipenem in mice infected with *Mab* results in immediate and remarkable reduction in lung CFU burden [39,45,47]. However, imipenem does not meet two out of the four criteria for an ideal positive comparator, as it is needs to be administered twice a day and via injection.

5. CONCLUSION

In summary, our studies demonstrated that 25 mg/kg of clofazimine, administered once daily via oral gavage, reduces *Mab* burden in the lungs of both C3HeB/FeJ and BALB/c mice over six weeks. Rifabutin, 20 mg/kg, once daily, administered by the same method exhibits bactericidal activity in C3HeB/FeJ mice but fails to reduce *Mab* lung burden in BALB/c mice over the six-week period. In addition to their efficacies against *Mab*, there were no noticeable adverse events in mice associated with these two drugs at the specified dosages. Based on these findings, we conclude that clofazimine meets the criteria for an ideal positive control and, therefore, propose that it be considered as comparator in future efforts to develop new drugs and regimens to treat *Mab* disease.

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ABBREVIATIONS

Mab	Mycobacteroides abscessus
CFZ	clofazimine
RFB	rifabutin

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HIGHLIGHTS

- Existing antibiotics to treat *M. abscessus* (*Mab*) disease are not optimal.
- There is an urgent need for new effective treatments for *Mab* disease.
- New treatments need to be compared with existing antibiotics.
- An existing antibiotic that can serve as a comparator is needed.
- Clofazimine has ideal attributes to serve as a positive control comparator.

IMPORTANCE

Mycobacteroides abscessus can cause life-threatening infections in patients with chronic lung conditions. New treatments are needed as cure rate using existing drugs is low. During pre-clinical phase of treatment development, it is important to compare the efficacy of the experimental drug against to existing ones with known history. Here, we demonstrate that clofazimine, one of the standard-of-care antibiotics used for treating *Mab* disease, can serve as a positive control comparator for efficacy assessments of experimental drugs and regimens to treat *M. abscessus* disease in mice.

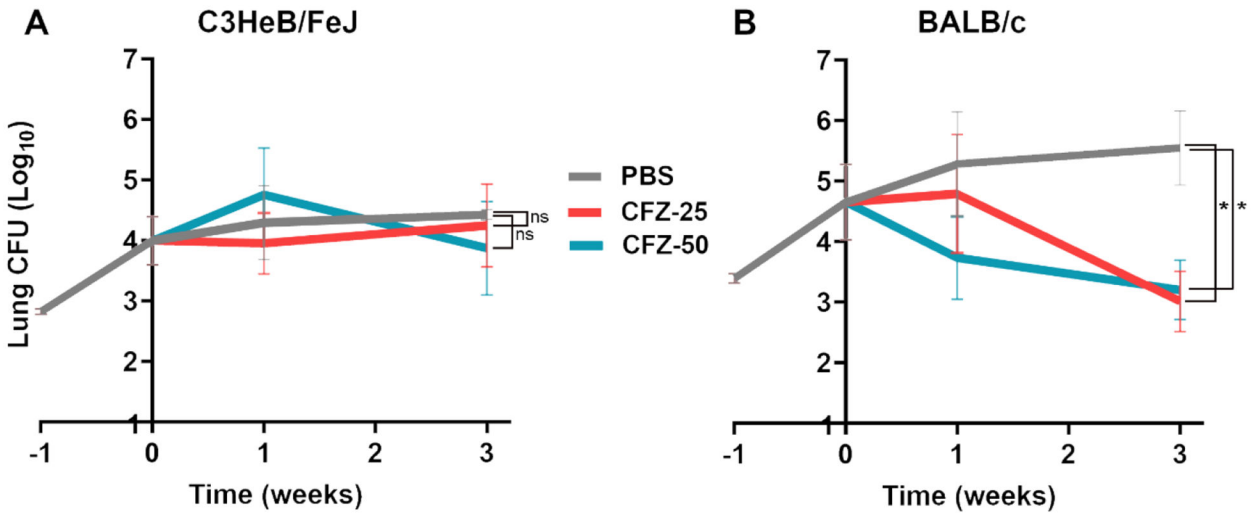

Figure 1. Burden of *M. abscessus* in the lungs of C3HeB/FeJ and BALB/c mice treated with clofazimine for three weeks.

Mean (±SEM) *Mab* lung burden ($\log_{10}$ CFU) in A) C3HeB/FeJ and B) BALB/c mice, n=5 per time-point, per treatment group, treated with 25 or 50 mg/kg clofazimine are shown in red and light blue, respectively. Mean (±SEM) *Mab* lung burden in the negative control group, administered with phosphate buffered saline (PBS), is shown in grey. Time-point week −1 represents the day following infection with *Mab*. Time-point week 0 represents the day antibiotic administration was initiated. CFZ-25, once daily 25 mg/kg clofazimine; CFZ-50, once daily 50 mg/kg clofazimine. P-values of PBS vs. each treatment group are represented as stars: * represents $p \leq 0.05$; 'ns' represents $p > 0.05$ (not significant).

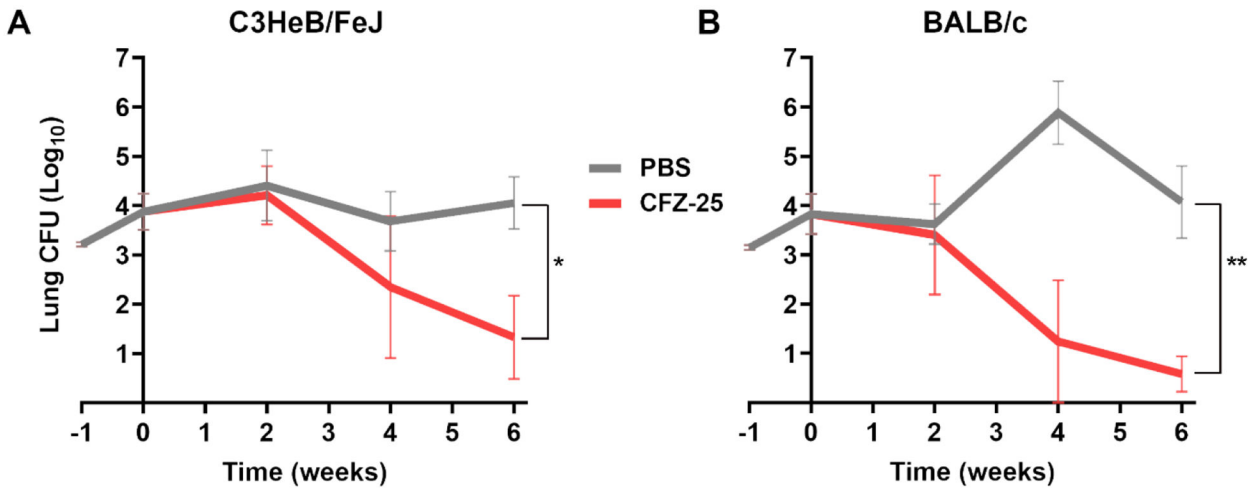

Figure 2. Burden of *M. abscessus* in the lungs of C3HeB/FeJ and BALB/c mice treated with 25mg/kg clofazimine for six weeks.

Mean (±SEM) *Mab* lung burden (log_{10} CFU) in A) C3HeB/FeJ and B) BALB/c mice, n=5 per time-point, per treatment group, treated with 25mg/kg clofazimine is shown in red. Mean (±SEM) *Mab* lung burden in the negative control group, administered with phosphate buffered saline (PBS), is shown in grey. Time-point week −1 represents the day following infection with *Mab*. Time-point week 0 represents the day antibiotic administration was initiated. CFZ-25, 25 mg/kg clofazimine. P-values of PBS vs each treatment group are represented as stars: * represents $p \leq 0.05$; ** represents $p \leq 0.01$.

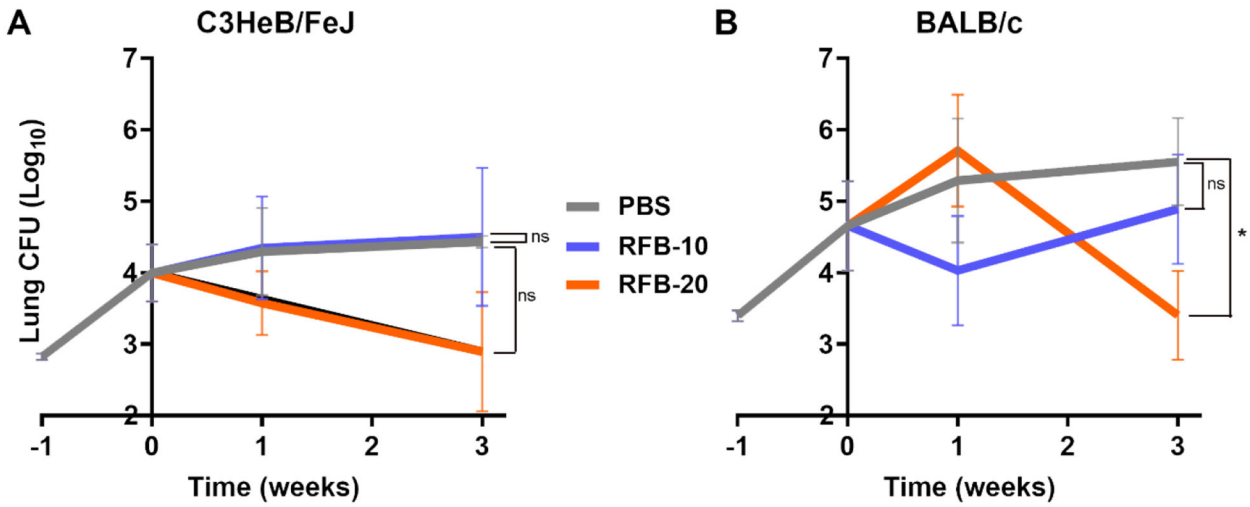

Figure 3. Burden of *M. abscessus* in the lungs of C3HeB/FeJ and BALB/c mice treated with rifabutin for three weeks.

Mean (±SEM) *Mab* lung burden ($\log_{10}$ CFU) in A) C3HeB/FeJ and B) BALB/c mice, n=5 per time-point, per treatment group, treated with 10mg/kg and 20 mg/kg rifabutin are shown in purple and orange, respectively. Mean (±SEM) *Mab* lung burden in the negative control group, administered with phosphate buffered saline (PBS), is shown in grey. Time-point week −1 represents the day following infection with *Mab*. Time-point week 0 represents the day antibiotic administration was initiated. RFB-10, 10mg/kg of rifabutin; RFB-20, 20mg/kg of rifabutin. P-values of PBS vs each treatment group are represented as stars: * represents $p \leq 0.05$; 'ns' represents $p > 0.05$ (not significant).

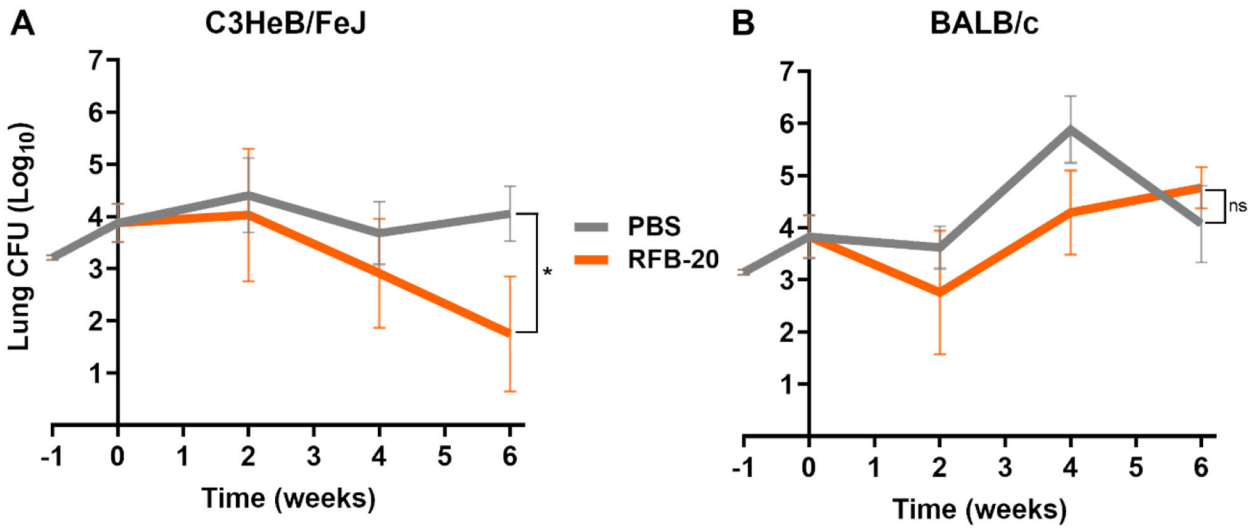

Figure 4. Burden of *M. abscessus* in the lungs of C3HeB/FeJ and BALB/c mice treated with 20mg/kg rifabutin for six weeks.

Mean (±SEM) *Mab* lung burden (log_{10} CFU) in A) C3HeB/FeJ and B) BALB/c mice, n=5 per time-point, per treatment group, treated with 20 mg/kg rifabutin is shown in orange. Mean (±SEM) *Mab* lung burden in the negative control group, administered with phosphate buffered saline (PBS), is shown in grey. Time-point week −1 represents the day following infection with *Mab*. Time-point week 0 represents the day antibiotic administration was initiated. RFB-20, 20mg/kg of rifabutin. P-values of PBS vs each treatment group are represented as stars: * represents p ≤0.05; 'ns' represents p>0.05 (not significant).

Table 1:

Selection of ideal positive comparator control from antibiotics considered for treating *Mab* disease [*]

Drug Name	Dosing Frequency	Adverse Events	Availability	Route of Administration
First-line antibiotics				
Amikacin	Once/Twice daily	High	Yes	Parenteral
Azithromycin [†]	Once daily	Moderate	Yes	Oral
Bedaquiline	Once daily	High	Yes	Oral
Cefazolin	>Twice daily	Low	Yes	Parenteral
Cefoxitin	>Twice daily	High	Yes	Parenteral
Clarithromycin [†]	Twice daily	High	Yes	Oral
Clofazimine	Once daily	Low	Yes	Oral
Imipenem	Twice daily	Low		Parenteral
Linezolid	Once/Twice daily	High	Yes	Oral
Omadacycline	Once daily	Low	[#]	Oral
Rifampicin	Once daily	Moderate	Yes	Oral
Tedizolid	Once daily	High	Yes	Oral
Tigecycline	Twice daily	Moderate	Yes	Parenteral
Additional antibiotics				
Ceftazidime	Twice daily	High	Yes	Oral
Ciprofloxacin	Once daily	High	Yes	Oral
Doxycycline	Twice daily	Moderate	Yes	Oral
Ethambutol	Twice daily	High	Yes	Oral
Minocycline	Twice daily	High	Yes	Oral
Moxifloxacin	Once daily	Low	Yes	Oral
Rifabutin	Once daily	Low	Yes	Oral
Trimethoprim/co-trimoxazole	Twice daily	High	Yes	Oral

Green color highlight indicates that antibiotic qualifies for the respective criteria

Red color highlight indicates disqualification

[†] induces macrolide resistance in *Mab subsp. abscessus and subsp. bolletii*

[#] Insufficent data

[*] Information presented in this table have been obtained from published references [16,17,21,88,90]. The information presented in this table reflect activities in human populations. Low adverse events refer to tolerable effects like gastrointestinal discomfort, mild diarrhoea, that allow these drugs to be suitable for longer treatment durations. Drugs exhibiting moderate and high adverse events refer to side-effects that are intolerable (acute and chronic)- like renal, hepatic, vestibular, or auditory impairment, congenital effects (in pregnant women), hypersensitivity reactions, seizure, neuropathy (optic/peripheral).