Characterization of Mouse Models of *Mycobacterium avium* Complex Infection and Evaluation of Drug Combinations

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The *Mycobacterium avium* complex is the most common cause of nontuberculous mycobacterial lung disease worldwide; yet, an optimal treatment regimen for *M. avium* complex infection has not been established. Clarithromycin is accepted as the cornerstone drug for treatment of *M. avium* lung disease; however, good model systems, especially animal models, are needed to evaluate the most effective companion drugs. We performed a series of experiments to evaluate and use different mouse models (comparing BALB/c, C57BL/6, nude, and beige mice) of *M. avium* infection and to assess the anti-*M. avium* activity of single and combination drug regimens, *in vitro*, *ex vivo*, and in mice. *In vitro*, clarithromycin and moxifloxacin were most active against *M. avium*, and no antagonism was observed between these two drugs. Nude mice were more susceptible to *M. avium* infection than the other mouse strains tested, but the impact of treatment was most clearly seen in *M. avium*-infected BALB/c mice. The combination of clarithromycin-ethambutol-rifampin was more effective in all infected mice than moxifloxacin-ethambutol-rifampin; the addition of moxifloxacin to the clarithromycin-containing regimen did not increase treatment efficacy. Clarithromycin-containing regimens are the most effective for *M. avium* infection; substitution of moxifloxacin for clarithromycin had a negative impact on treatment efficacy.

Pulmonary disease due to nontuberculous mycobacteria (NTM) is on the rise worldwide (1, 2), and in several areas, including the United States and Canada, the incidence rate of such disease is higher than that of tuberculosis (3, 4). The most common cause of pulmonary NTM disease is infection with bacilli from the *Mycobacterium avium* complex (MAC). Although presentation and severity of MAC infections are varied and poorly understood, progressive cavitary disease can lead to extensive lung destruction and respiratory failure within a few years if left untreated (5).

Unfortunately, treatment of pulmonary MAC infection is not straightforward. As with other NTM infections, the evidence base for treatment is very limited, and current treatment guidelines are largely based on expert opinion and a limited number of small, and sometimes contradictory, studies. The effectiveness of the macrolide clarithromycin has clearly been established for MAC infections; however, this drug must be given as part of a combination regimen to prevent the selection of clarithromycin-resistant bacilli (5, 6). The most complementary and efficacious companion drugs, as well as the optimal regimen and duration of the multidrug administration, are unknown. The current therapy guidelines for MAC lung disease recommend the combination of a macrolide (clarithromycin or azithromycin) with ethambutol and a rifamycin (usually rifampin), with or without an initial phase including an aminoglycoside (streptomycin or amikacin), administered for a minimum of 12 months after sputum culture conversion; this recommended regimen is considered successful in approximately 50 to 60% of patients (5). However, administration of the macrolide-ethambutol-rifamycin-aminoglycoside combination is often confounded by drug toxicities and poor tolerability. Thus, there is a clear need for the development of an optimal treatment strategy for MAC lung disease.

A major hindrance in the development of an optimized treatment regimen is the lack of reliable and standardized *in vitro* and

in vivo experimental models of MAC chemotherapy. The methods and findings of reported studies have been extremely varied, often yielding discrepant results. As a step toward improving the experimental tools available for MAC chemotherapy studies, we have systematically evaluated a series of drugs and drug combinations for *in vitro* activity against *M. avium*. In addition, we performed side-by-side comparisons of different mouse strains to determine an optimal *in vivo* model for MAC chemotherapy experiments. Our work provides a framework for the testing of drugs and drug combinations against MAC organisms and additionally clarifies some of the previous inconsistencies about the utility of different drugs, especially moxifloxacin, in the treatment of MAC disease.

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MATERIALS AND METHODS

Bacterial culture. *M. avium* strain Chester (also designated MAC 101, ATCC 700898) was used in all experiments. This strain has previously been demonstrated to multiply to high numbers in beige mice, allowing for assessment of treatment (7). The bacteria were passaged in mice, frozen in 1-ml aliquots, and stored at -80°C before use; this method has

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been shown to maintain *in vivo* virulence of *M. avium* (8). For each experiment, an aliquot was thawed and subcultured at 37°C in Middlebrook 7H9 broth (Difco, Detroit, MI, USA) supplemented with 10% (vol/vol) oleic acid-albumin-dextrose (OADC [Difco]) and 0.05% (vol/vol) Tween 80 (Sigma, St. Louis, MO, USA). *M. avium* cultures were incubated for 4 weeks before use in an experiment or infection.

Animals. Animal experiments were performed with 6-week-old female mice. Swiss, BALB/c, and nude mice were purchased from Charles River (Wilmington, MA, USA), and C57BL/6 and beige mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All reported research was approved by the Johns Hopkins University Animal Care and Use Committee (ACUC), and animals were cared for in accordance with the ACUC guidelines of the university.

Antimicrobials. Moxifloxacin, rifampin, rifapentine, amikacin, and ethambutol were purchased from Sigma. Stock solutions of these drugs were prepared by dissolving them in sterile water. Clarithromycin was donated by Abbott Laboratories (Abbott Park, IL, USA), and linezolid was donated by Pfizer (Groton, CT, USA). Stock solutions of these two drugs were prepared by first dissolving them in dimethyl sulfoxide (DMSO) and subsequently diluting them in sterile water. All stock solutions were stored at 4°C for up to 1 week.

MIC assays. For MIC determination on solid media, drugs were incorporated into 7H11 agar (Difco) supplemented with 10% OADC to obtain 2-fold concentration dilutions (in mg/liter) of 0.06 to 4 for moxifloxacin, 0.25 to 16 for rifampin, 0.06 to 4 for rifapentine, 2 to 32 for amikacin, 1 to 32 for ethambutol, 0.03 to 4 for clarithromycin, and 0.5 to 8 for linezolid. As acidic pH has a negative impact on the *in vitro* potency of macrolides and aminoglycosides (9, 10), clarithromycin and amikacin were also incorporated into 10% OADC-enriched Mueller-Hinton agar (pH 7.3; Difco) in addition to 7H11 agar (pH 6.6). The turbidity of the *M. avium* culture was adjusted with sterile phosphate-buffered saline to an optical density at 600 nm (OD_{600}) of 1.0. Then, 0.5 ml of 10^{-3} and 10^{-5} culture dilutions was inoculated onto control (drug-free) and drug-containing agar plates, which were then incubated for 28 days at 37°C. The MIC was defined as the lowest concentration that inhibited $\geq 99\%$ of the CFU count compared with the CFU count for the control medium. Each MIC test was performed in duplicate.

For MIC determination in liquid media, serial 2-fold dilutions of each drug (at the same range of concentrations used for MIC assays on solid media) were prepared in 2.5 ml of 7H9 broth supplemented with 10% OADC but without Tween 80. Drug-free and drug-containing broth aliquots were then inoculated with 0.1 ml of the same broth culture that was used for MIC determination on 7H11 agar. After 14 days of incubation at 37°C, when growth was visible in the drug-free control cultures, the MIC was defined as the lowest drug concentration that completely inhibited bacterial growth as determined by the naked eye. Each MIC test was performed in duplicate.

MBC assay. For each drug, the liquid media in the MIC culture tubes that did not exhibit any visible growth were plated on 7H11 agar supplemented with 10% OADC to determine CFU counts after 4 weeks of incubation at 37°C. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of drug that killed $\geq 99\%$ of the CFU compared with the CFU count of the initial inoculum used for the MIC assays.

FICI and fractional bactericidal concentration index (FBCI). The combined activities of rifampin-ethambutol, rifapentine-ethambutol, moxifloxacin-ethambutol, and moxifloxacin-clarithromycin against *M. avium* were studied by the checkerboard titration technique (11) using 7H9 broth supplemented with 10% OADC but without Tween 80. Serial 2-fold dilutions of each drug, ranging from 0.25 to 4.0 times the MIC, were prepared in 2.5 ml broth, which was inoculated (along with drug-free broth) with 0.1 ml of *M. avium* culture suspension at an OD_{600} of 1.0. The inoculated samples were incubated for 14 days at 42°C, and the MIC for each drug combination was defined as the lowest concentration that completely inhibited bacterial growth as determined by the naked eye.

These results were used to calculate the fractional inhibitory coefficient index (FICI) as follows: $FICI = (MIC_{\text{drug A in combination}}/MIC_{\text{drug A alone}}) + (MIC_{\text{drug B in combination}}/MIC_{\text{drug B alone}})$. Combinations were considered synergistic if the FICI was ≤ 0.5 , antagonistic if the FICI was ≥ 4.0 , and to have no interaction if the FICI was > 0.5 but < 4.0 .

To determine the FBCI, the MBC of drug combinations was calculated by determining CFU counts (on 7H11 agar supplement with 10% OADC) from the broth vials with no visible growth in the MIC assay. MBC values were used to calculate the FBCI in the same way as the FICI was calculated, and the results were interpreted according to the definitions of synergism, indifference, and antagonism, as for the FICI.

Ex vivo study: SIT and SBT. Sixty outbred Swiss mice were randomized into four subgroups of 15 mice, and each group received a single pulse of the following drug combinations: clarithromycin-ethambutol-rifampin, clarithromycin-ethambutol-rifampin-amikacin, moxifloxacin-ethambutol-rifampin, and moxifloxacin-ethambutol-rifampin-amikacin. Drug doses were as follows: 10 mg/kg of body weight for rifampin and 100 mg/kg for clarithromycin, moxifloxacin, ethambutol, and amikacin. All drugs were administered in a volume of 0.2 ml by esophageal cannula (gavage) except amikacin, which was administered by subcutaneous injection. Rifampin was given 1 h before the other drugs to avoid possible adverse pharmacokinetic interactions (12, 13). Two hours after rifampin administration (i.e., 1 h after administration of the other drugs), mice were anesthetized with isoflurane and exsanguinated by cardiac puncture. Blood was collected in sterile microcentrifuge tubes and rested for 30 min at room temperature before centrifugation for serum separation. The individual serum samples from each treatment group were then pooled. For each group, 2.5 ml of the pooled serum was combined with 2.5 ml of 7H9 broth with 10% OADC but without Tween 80 to create the 1/2 dilution and then underwent further 2-fold dilutions to obtain the 1/4, 1/8, and 1/16 serum dilutions. All diluted serum samples and controls (prepared with serum from mice who did not receive any drug) were inoculated with 0.1 ml of the same mycobacterial suspension at an OD_{600} of 1.0 that was used to determine MIC and MBC. The inoculated (and no-bacterium control) samples were incubated for 14 days at 37°C, when growth was visible in the drug-free control cultures. The serum inhibitory titer (SIT) was defined as the highest serum dilution that completely inhibited bacterial growth as determined by the naked eye. For serum bactericidal titer (SBT) determination, the liquid media in the SIT culture tubes that did not exhibit any visible growth were plated on 7H11 agar supplemented with 10% OADC to determine CFU counts after 4 weeks of incubation at 42°C. The SBT was defined as the lowest concentration of drug that killed $\geq 99\%$ of the CFU compared with the CFU count of the initial inoculum used for the SIT assays.

Mouse model comparison. *M. avium* infection was assessed in four strains of mice: BALB/c, C57BL/6, athymic *nu/nu* mice (nude), and beige; for each strain, 30 mice were simultaneously aerosol infected with *M. avium* using a 7H9 broth containing $9.48 \log_{10}$ CFU/ml using the inhalation exposure system (Glas-Col Inc., Terre Haute, IN, USA). Each mouse was weighed weekly from the day after infection to the day of its sacrifice. Three animals from each strain were sacrificed on day 1 and at weeks 1, 2, 3, 4, 8, 12, and 23 postinfection, and lungs and spleens were examined for gross lesions. To compare the usefulness of each strain as the model for MAC chemotherapy, we initiated clarithromycin treatment for 10 mice from each strain group, starting 4 weeks after infection. Clarithromycin at 100 mg/kg was administered in a 0.2-ml volume by esophageal cannula (gavage) 5 days per week. These drug doses represent the equipotent doses administered to humans (14–18). Three mice from each group were sacrificed after 1 and 2 months of treatment (i.e., at weeks 8 and 12 postinfection), and lungs and spleens were examined for gross lesions. A small piece of each lung was removed, formalin fixed, sectioned, and stained with Ziehl-Neelsen for acid-fast bacillus (AFB) detection. All lungs and spleens were then homogenized in sterile phosphate-buffered saline, and diluted and serially diluted homogenates were plated in duplicate for CFU counting on selective 7H11 agar plates (7H11 agar supplemented with

TABLE 1 Single-drug MIC and MBC values for *M. avium* in relation to the reported $C_{\max}$ value in humans

Drug ^a	MIC (mg/liter)		MBC (mg/liter)	$C_{\max}$ (mg/liter) ^b
	7H11 agar	7H9 broth		
RIF	8	>16	>16	10
RFP	2	>4	>4	10
CLR	2	>4	>4	2.5–3
MXF	2	2	2	3–4
EMB	32	16	16	3–4
AMK	16	16	ND	20–40
LZD	16	ND ^c	ND	13–18

^a RIF, rifampin; RFP, rifapentine; CLR, clarithromycin; MXF, moxifloxacin; EMB, ethambutol; AMK, amikacin; LZD, linezolid.

^b Peak serum concentration ($C_{\max}$) values were obtained from references 14 to 20.

^c ND, not determined.

10% OADC and containing 50 µg/ml cycloheximide, 100 µg/ml carbenicillin, 200 U/ml polymyxin B, and 20 µg/ml trimethoprim. Plates were incubated for 4 weeks at 37°C before CFU enumeration.

Combination therapy in BALB/c and nude mice. Fifty-five BALB/c and 55 nude mice were simultaneously aerosol infected with *M. avium* by using 7H9 broth containing $6.39 \log_{10}$ CFU/ml by using the inhalation exposure system. Following infection, the mice from each strain were randomized into 4 subgroups, and 28 days postinfection, treatment was initiated with one of the following drug combinations: clarithromycin-ethambutol-rifampin (10 mice from each strain), moxifloxacin-ethambutol-rifampin (10 mice from each strain), clarithromycin-moxifloxacin-ethambutol-rifampin (10 mice from each strain), and no treatment (25 mice from each strain). Daily drug doses were 10 mg/kg for rifampin and 100 mg/kg for clarithromycin, moxifloxacin, and ethambutol. All drugs were administered in a total volume of 0.2 ml by esophageal cannula; rifampin was given 1 h before administration of the other drugs to avoid possible adverse pharmacokinetic interactions (12, 13). Five animals from the untreated group were sacrificed the day after infection and on the day of treatment initiation to determine CFU counts implanted and pretreatment, respectively. Five animals from both treated and untreated groups were sacrificed 8 and 16 weeks after infection (after 4 and 12 weeks of drug administration, respectively, for the treated animals). Lungs were examined for gross lesions and were homogenized and plated for CFU count determination as described above.

Statistical analyses. CFU counts were $\log_{10}$ transformed before analysis. The group means in the combination therapy experiment were compared by two-way analysis of variance with Tukey's multiple-comparison test. All analyses were performed with GraphPad Prism version 6.05.

RESULTS

Single-drug MIC and MBC values for *M. avium*. The MICs of rifampin, rifapentine, clarithromycin, moxifloxacin, ethambutol, amikacin, and linezolid for *M. avium*, as well as the associated MBC values, are presented in Table 1. For the rifamycins, clarithromycin and linezolid, the MIC values measured in liquid me-

TABLE 3 SIT and SBT values of multidrug combinations for *M. avium*

Drug combination	Serum dilution ^a	
	SIT	SBT
Clarithromycin-ethambutol-rifampin	1/16	1/4
Clarithromycin-ethambutol-rifampin-amikacin	1/4	1/2
Moxifloxacin-ethambutol-rifampin	1/4	1/2
Moxifloxacin-ethambutol-rifampin-amikacin	1/8	1/8

^a Sera were obtained from Swiss mice after one dose of each of the drug combinations.

dium (7H9 broth) were at least one dilution above the values measured on solid medium (7H11 agar). For the drugs most commonly recommended to treat MAC infection (clarithromycin, rifampin, ethambutol, and amikacin), the MIC values were close to or greater than the peak serum concentration obtained at the standard doses in humans (19–25). The MIC value for clarithromycin was one dilution lower on Mueller-Hinton agar (pH 7.3) than on 7H11 agar (pH 6.6), i.e., 1 mg/liter compared to 2 mg/liter, while the MIC value for amikacin was 16 mg/liter on both types of agar. For those drugs in which the MBC was determined, only moxifloxacin had an MBC value close to the peak human serum concentration, whereas rifampin, ethambutol, and clarithromycin had MBC values higher than the peak human serum concentrations (Table 1), thus indicating that none of these drugs alone was bactericidal for *M. avium*.

FICI and FBCI for two-drug combinations. The checkerboard analysis of two-drug combinations demonstrated that rifampin-ethambutol, rifapentine-ethambutol, and moxifloxacin-ethambutol had synergistic bacteriostatic activities against *M. avium*, while only rifapentine-ethambutol had synergistic bactericidal activity (Table 2). The MIC of rifampin alone was >16 mg/liter, but the MIC of rifampin when combined with ethambutol was 4 mg/liter. The FICI interpretation for the moxifloxacin-clarithromycin combination was no interaction, even though the MIC of clarithromycin alone was higher than 4 mg/liter, and when clarithromycin was combined with moxifloxacin the MIC was 0.125 mg/liter. No antagonism was observed between clarithromycin and moxifloxacin.

Ex vivo SIT and SBT for multidrug combinations. The serum bacteriostatic and bactericidal activities of clinically relevant drug combinations (i.e., those combination often administered to patients with MAC infection) were determined after drug administration in outbred Swiss mice. This mouse strain was used (as opposed to an inbred mouse strain) to allow for genetic differences in drug metabolism, as in outbred (human) hosts. The most active drug combination in mouse sera was clarithromycin-ethambutol-rifampin, which inhibited the growth of *M. avium* up to a serum dilution of 1/16 (Table 3).

TABLE 2 MIC and MBC values and the associated FICI and FBCI of two-drug combinations for *M. avium*

Drug combination ^a	FICI			FBCI		
	MIC (mg/liter)	Value	Interpretation	MBC (mg/liter)	Value	Interpretation
RIF-EMB	4/2	<0.325	Synergism	>16/2	2	No interaction
RFP-EMB	0.5/4	<0.325	Synergism	4/1	<0.5	Synergism
MXF-EMB	2/0.5	0.325	Synergism	2/2	0.625	No interaction
MXF-CLR	2/0.125	1.1	No interaction	2/0.125	1.1	No interaction

^a RIF, rifampin; RFP, rifapentine; CLR, clarithromycin; MXF, moxifloxacin; EMB, ethambutol.

TABLE 4 Log₁₀ CFU of *M. avium*/lung in four strains of mice with and without clarithromycin treatment from week 4 to week 12 postinfection

Mouse strain and treatment	Mean log ₁₀ CFU/lung (SD) by time point postinfection							
	Day 1	Wk 1	Wk 2	Wk 3	Wk 4	Wk 8	Wk 12	Wk 23
BALB/c								
Untreated	4.92 (0.02)	5.17 (0.26)	5.68 (0.10)	5.32 (0.07)	7.03 (0.35)	10.60 (0.82)	8.79 (0.44)	6.13 (0.95)
Clarithromycin						7.95 (0.07)	4.46 (0.15)	
C57BL/6								
Untreated	4.98 (0.01)	5.16 (0.13)	5.72 (0.09)	6.39 (0.14)	7.15 (0.15)	8.61 (0.27)	8.51 (0.21)	6.74 (0.18)
Clarithromycin						8.00 (0.01)	5.69 (0.08)	
Beige								
Untreated	4.88 (0.14)	5.35 (0.20)	5.89 (0.02)	5.77 (0.20)	7.36 (0.05)	11.30 (0.01)	9.30 (0.52)	7.54 (0.25)
Clarithromycin						7.91 (0.07)	5.28 (0.44)	
Nude								
Untreated	5.01 (0.06)	5.34 (0.28)	5.60 (0.21)	5.54 (0.16)	7.26 (0.23)	11.20 (0.20)	10.90 (0.41)	7.40 (0.90)
Clarithromycin						7.89 (0.17)	6.16 (1.00)	

Comparison of MAC infection in different mouse strains. Four weeks after aerosol infection of the immunocompetent (BALB/c and C57BL/6) and the immune-deficient (nude and beige) mouse strains, the lung CFU counts of *M. avium* increased by around 2 log₁₀ CFU, and by week 8 postinfection, the lung bacterial load had increased by an additional 4 log₁₀ CFU in all strains except for C57BL/6 mice, in which the lung CFU counts increased by 1.5 log₁₀ (Table 4). Between weeks 8 and 12 postinfection, the bacterial load either plateaued (C57BL/6 and nude mice) or decreased (BALB/c and beige mice). In all four mouse strains, *M. avium* disseminated to the spleen (Table 5). During the entire 23-week course of *M. avium* infection, none of the mice in any group appeared sick, and none died. The body and spleen weights in all groups steadily increased, even in the nude mice. The ratio of spleen to body weight remained constant, in the range of 0.50 to 0.75% in all untreated mice. Lung gross pathology at 12 weeks postinfection is presented in Fig. 1; all of the untreated mice exhibited gross lung lesions. AFB were visible in

lung sections from all strains of mice (Fig. 2); however, the lung sections from the infected BALB/c and nude mice exhibited aggregates of AFB that were not visible in the lung sections from the other mouse strains. Administration of clarithromycin was initiated 4 weeks postinfection. The activity of this drug was delayed, but during the second month of treatment the bacterial burden was significantly reduced (by 2 log₁₀ CFU) in the clarithromycin-treated mice compared to that in the untreated controls in all four mouse strains (Table 4). The impact of clarithromycin treatment on lung gross pathology is shown in Fig. 1. All of the treated mice, regardless of strain, exhibited a reduction in gross lesions compared to their untreated counterparts, and AFB were not visible in any of the treated mice. **Multidrug combination therapy in MAC-infected BALB/c and nude mice.** Our data indicated that the nude mouse was highly susceptible to *M. avium* aerosol infection, and the impact of treatment on the lung infection could be clearly evaluated in the BALB/c mouse. Therefore, we used both of these strains of mice to analyze clinically relevant multidrug combination regimens for treatment of *M. avium* infection. Fifty-five BALB/c and 55 nude mice were simultaneously aerosol infected with *M. avium*, and approximately 5.1 and 5.2 log₁₀ CFU were implanted in the lungs of BALB/c and nude mice, respectively. After infection, mice from each strain were randomized into one of the following treatment groups: (i) no treatment, (ii) clarithromycin-ethambutol-rifampin, (iii) moxifloxacin-ethambutol-rifampin, or (iv) clarithromycin-moxifloxacin-ethambutol-rifampin. Treatment was initiated 4 weeks after infection, when the bacterial burden was around 6.4 and 6.8 log₁₀ CFU in BALB/c and nude mice, respectively (Table 6). In untreated mice, the lung CFU counts then plateaued at around 7 log₁₀ CFU in the BALB/c mice and at nearly 9 log₁₀ CFU in the nude mice, which were maintained for the 16-week duration of the experiment. The most efficacious regimens in both strains of mice were the clarithromycin-containing regimens, while the moxifloxacin-ethambutol-rifampin regimen was less effective at killing *M. avium* (in both strains of mice, lung CFU counts were statistically significantly lower in the mice treated with the clarithromycin-free regimen than in the mice treated with either of the clarithromycin-containing regimens, *P* ≤

TABLE 5 Log₁₀ CFU of *M. avium*/spleen in four strains of mice with and without clarithromycin treatment from week 4 to week 12 postinfection

Mouse strain and treatment	Mean log ₁₀ CFU/spleen (SD) by time point postinfection			
	Wk 2	Wk 4	Wk 8	Wk 12
BALB/c				
Untreated	2.31 (0.32)	2.91 (0.28)	7.95 (0.07)	5.64 (0.18)
Clarithromycin			2.65 (0.36)	1.09 (0.08)
C57BL/6				
Untreated	2.70 (0.29)	4.24 (0.92)	8.00 (0.01)	6.03 (3.40)
Clarithromycin			3.74 (0.59)	3.40 (0.09)
Beige				
Untreated	2.46 (0.28)	3.80 (0.69)	7.91 (0.07)	5.39 (0.19)
Clarithromycin			4.29 (0.67)	3.05 (0.51)
Nude				
Untreated	2.00 (0.61)	2.91 (0.69)	7.91 (0.07)	5.39 (0.19)
Clarithromycin			4.29 (0.67)	3.05 (0.51)

FIG 1 Gross lung pathology in four strains of mice, 12 weeks after aerosol infection with *M. avium* (implantation of approximately $5 \log_{10}$ CFU/lung). Four weeks postinfection, clarithromycin (100 mg/kg/day) was administered to a subgroup of mice.

0.0001). The addition of moxifloxacin to the clarithromycin-ethambutol-rifampin combination did not improve the efficacy of the regimen, but no antagonism was observed in our experiment. The impacts of the drug regimens on *M. avium* infection were similar in both strains of mice but more pronounced in the BALB/c strain.

DISCUSSION

M. avium is the most common NTM isolated in Europe and the United States (1, 5). The major limitations to effective therapy for *M. avium* lung disease are the lack of antimicrobial drugs with low toxicity and high bactericidal activity. Clarithromycin is the cornerstone of *M. avium* treatment, but the most complementary and efficacious companion drugs to combine with clarithromycin for multidrug therapy remain to be determined. The MIC of moxifloxacin for *M. avium* has been reported to be relatively low and below the serum concentration of the drug that is achieved with the standard dosing in humans (19, 26), suggesting the drug could be active against *M. avium* *in vivo*. However, there have been conflicting and confusing data regarding the efficacy of moxifloxacin for MAC treatment, both in patients and in experimental settings (26–28). Therefore, we conducted a series of experiments to char-

acterize the bacteriostatic and bactericidal activities of antimicrobial drugs, especially moxifloxacin, *in vitro* and *ex vivo*, and to evaluate and use different mouse models of *M. avium* infection and chemotherapy. It should be noted that the objective of the *in vivo* work was not to develop an experimental model of MAC disease but rather to identify the mouse model(s) that can serve as a preclinical tool for the evaluation of drug regimens for MAC treatment.

Our data confirmed the already well-established natural resistance of *M. avium* to most of the indicated antibiotics (when used alone), with the MIC and MBC values for most drugs being equal to or greater than the maximum concentration achievable during treatment of patients (Table 1). However, when examining two-drug combinations, we observed synergistic bacteriostatic activities of the rifampin-ethambutol, rifapentine-ethambutol, and moxifloxacin-ethambutol pairings. After evaluation of infection in four different strains of mice (BALB/c, C57BL/6, beige, and nude), our data indicate that nude mice are highly susceptible to aerosol infection with *M. avium*; however, the impact of antibiotic treatment was most clearly observed in the BALB/c mouse model of infection. Based on the assessment of three different multidrug treatment regimens in *M. avium*-infected BALB/c and nude mice, our data indicate that moxifloxacin is not beneficial when combined with clarithromycin or other drugs against *M. avium* *in vivo*.

Our work also highlights the influence of experimental conditions on MIC determination, as the MICs of several antibiotics for *M. avium* differed when measured using liquid and solid media (Table 1), and the MIC of clarithromycin was also affected by the pH of the solid media, with the MIC value being lower on Mueller-Hinton than on the more acidic 7H11 agar, confirming previous findings (10). Our MIC data for moxifloxacin differs from what has been previously reported; we found the MIC for *M. avium* to be 2 mg/liter, whereas Kohno and colleagues found the MIC to be 1 mg/liter (26). In addition, Kohno and colleagues also reported antagonism between moxifloxacin and clarithromycin, which we did not observe in our experiments (Table 2). Although the same liquid medium was used in our assays (7H9 broth with OADC and without Tween 80), Kohno and colleagues used the microdilution 96-well microplate method (using 200 μ l of culture broth with an inoculum of $1.0 \times 10^5 \log_{10}$ CFU per well) to determine the MIC values, while we used vials containing 2.5 ml broth with an inoculum of $6.6 \times 10^6 \log_{10}$ CFU per vial. Likely, the differences we observed between MIC and FICI values result from differences in experimental conditions. However, in our hands, the MIC and MBC values of moxifloxacin for *M. avium* were high, with a peak serum value-to-MIC ratio always lower than 10, suggesting even

FIG 2 Lung histopathology in four strains of mice, 12 weeks after aerosol infection with *M. avium*. Lung sections from untreated mice were stained with Ziehl-Neelsen, and red arrowheads indicate AFB. Images were captured at $\times 50$ (BALB/c) or $\times 100$ (C57BL/6, beige, and nude) magnification.

TABLE 6 Log₁₀ CFU of *M. avium*/lung in BALB/c and nude mice with and without multidrug treatment from week 4 to week 16 postinfection

Mouse strain and treatment ^c	Mean log ₁₀ CFU/lung (SD) by time point postinfection				
	Day 1	Wk 2	Wk 4	Wk 8 ^a	Wk 16 ^b
BALB/c					
Untreated	5.14 (0.25)	5.86 (0.18)	6.39 (0.16)	6.90 (0.24)	7.08 (0.26)
CLR-EMB-RIF				5.50 (0.07)	4.87 (0.07)
MXF-EMB-RIF				5.56 (0.09)	5.79 (0.16)
CLR-MXF-EMB-RIF				5.53 (0.13)	4.68 (0.57)
Nude					
Untreated	5.26 (0.04)	6.25 (0.03)	6.79 (0.57)	8.94 (0.51)	8.84 (0.34)
CLR-EMB-RIF				5.92 (0.42)	6.00 (0.18)
MXF-EMB-RIF				6.62 (0.49)	7.65 (0.60)
CLR-MXF-EMB-RIF				6.34 (0.20)	5.86 (0.16)

^a At week 8 (after 1 month of treatment) in both BALB/c and nude mice, the lung CFU counts in all treated groups were statistically significantly different from those in the untreated mice ($P \leq 0.0001$ for each group).

^b At week 16 (after 2 months of treatment) in both BALB/c and nude mice, the lung CFU counts in all treated groups were statistically significantly different from those in the untreated mice ($P \leq 0.0001$ for each group); the lung CFU counts were also statistically significant between either the CLR-EMB-RIF or CLR-MXF-EMB-RIF group and the MXF-EMB-RIF group ($P \leq 0.0001$).

^c CLR, clarithromycin; EMB, ethambutol; RIF, rifampin; MXF, moxifloxacin.

from the *in vitro* work that moxifloxacin may not be useful for the treatment of *M. avium*.

To our knowledge, we report here the first comparison study of different mouse models of *M. avium* aerosol infection. Of the few studies that have evaluated mouse models, the infections were done primarily by intravenous injection (7, 29–31). Contrary to the mouse model of tuberculosis in which untreated mice infected by aerosol with more than 3.5 log₁₀ CFU implanted in the lungs die 4 to 6 weeks after infection (32), none of the mice infected by aerosol with a lung implantation of more than 6.5 log₁₀ CFU died or even appeared to be sick, despite impressive gross lung pathology (Fig. 1). These findings reflect the low virulence of *M. avium*, which is an opportunistic pathogen in patients made vulnerable by underlying disease. Importantly, the bacterial burden in the mouse lungs that we achieved through aerosol infection, ranging from 6.39 to 7.36 log₁₀ per lung at treatment initiation, adequately (and conservatively) represents the bacterial burden in patients with MAC pulmonary disease, which has been shown to range from 1.48 to 6.84 log₁₀ CFU/ml of bronchoscopic microsampling or lavage sample (33), indicating that our assessment of chemotherapy was in an infection model with an appropriate bacterial burden.

In order to evaluate different strains of mice as models for conducting MAC experimental chemotherapy studies, our primary variable was the mouse strain, and to test these different mice, we used one bacterial strain, *M. avium* Chester, known to actively multiply in mice (7), as well as standard drug doses that represent and have been validated to be the equipotent doses (based on relevant pharmacokinetic parameters for each drug) that would be administered to humans (14–18). A previous study found that treatment outcomes in beige mice infected with MAC strains isolated from patients with systemic MAC disease did not correlate with treatment outcomes in those patients (comparing the decrease in liver and spleen CFU counts in mice to the decrease in blood CFU counts in patients) (34); however, in this study, the drug doses of the mice and patients were not equipotent, making true comparison impossible. We hope that using human equipotent doses in mice will increase the usefulness of our models. The mouse strains we selected represented both immunocompetent

(BALB/c and C57BL/6) and immune-deficient (beige and nude) strains that have been successfully used as models for the chemotherapy of other mycobacterial diseases. In this study, the BALB/c mice provided the best model for the assessment of MAC chemotherapy, and this model could be used to assess the impact of the MAC bacterial strain on the antimicrobial activity of treatment regimens. Recently, C3HeB/FeJ (commonly referred to as “Kramnik”) mice have been shown to develop necrotic hypoxic lesions with caseation (35, 36), and the use of these mice as a new model for the assessment of antituberculosis drugs is growing; it would be interesting to assess the potential of this mouse strain as a model for MAC chemotherapy as well.

Although we found the nude and BALB/c mice to be the most appropriate (of the mouse strains tested) as the model system to evaluate susceptibility to *M. avium* aerosol infection and *M. avium* chemotherapy, respectively, we found that in all four mouse strains studied, clarithromycin was the key drug in the treatment regimens, thus confirming the place of clarithromycin as the cornerstone drug in treatment of MAC lung disease. Our data suggest that moxifloxacin should not be used as a replacement for clarithromycin for the treatment of *M. avium* infection if the bacteria are susceptible to clarithromycin.

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