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Time–kill kinetics of antibiotics active against rapidly growing mycobacteria

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Objectives: This study was conducted to generate basic pharmacodynamic information on the relationship between antibiotic concentrations and the growth of rapidly growing mycobacteria (RGM), and thereby contribute to a better understanding of current and future drug regimens for diseases caused by RGM.

Methods: Type strains of *Mycobacterium abscessus* and *Mycobacterium fortuitum* were used; the MICs of cefoxitin, amikacin, moxifloxacin, linezolid and clarithromycin were determined by broth microdilution. Time–kill assays were performed, exposing the bacteria to 2-fold concentrations from 0.25 to 32 times the MIC at 30°C for 120 h. The sigmoid maximum effect (E_{max}) model was fitted to the time–kill curves data.

Results: The highest killing of *M. abscessus* was observed between 24 and 72 h; amikacin had the highest E_{max} (0.0427 h^{-1}), followed by clarithromycin (0.0231 h^{-1}) and cefoxitin (0.0142 h^{-1}). For *M. fortuitum*, between 3 and 24 h, amikacin also showed the highest E_{max} (0.1933 h^{-1}). There were no significant differences between the Hill's slopes determined for all the antibiotics tested against *M. abscessus* or *M. fortuitum* ($P=0.2213$ and $P=0.2696$, respectively).

Conclusions: The total effect observed for all antibiotics was low and primarily determined by the E_{max} and not by the Hill's slope. The limited activity detected fits well with the poor outcome of antibiotic treatment for disease caused by RGM, particularly for *M. abscessus*. An evaluation of drug combinations will be the next step in understanding and improving current treatment standards.

Keywords: pharmacodynamics, non-tuberculous mycobacteria, *Mycobacterium abscessus*, kill rate, E_{max} model

Introduction

Infections caused by non-tuberculous mycobacteria (NTM) are emerging in many settings, particularly those where the incidence of tuberculosis is in decline.[1] NTM are environmental organisms, able to produce chronic disease in patients with a local or systemic immune impairment. Pulmonary NTM disease is the most frequent manifestation, involving both slowly growing mycobacteria and rapidly growing mycobacteria (RGM), with a variation in the epidemiology by geographical region.[2]

The treatment of such diseases is complicated, as NTM are naturally resistant to most commonly used antibiotics and the outcome is often poor. In RGM disease, *Mycobacterium abscessus* disease is the most frequent but also the most difficult to treat. Currently recommended treatment regimens for *M. abscessus* depend on the infecting subspecies. For *M. abscessus* subspecies *abscessus* and *M. abscessus* subspecies *bolletii* strains that show inducible macrolide resistance, a combination of three or four drugs is used that includes amikacin, cefoxitin, imipenem, tigecycline or linezolid, while for *M. abscessus* subspecies *bolletii* strains that lack inducible macrolide resistance (former *Mycobacterium massiliense*), the recommended regimens include a macrolide in combination with two drugs from amikacin, cefoxitin, imipenem and linezolid. The choice of drugs is based on *in vitro* drug susceptibility testing. The treatment should continue for more than 12 months after cultures have converted to negative.[3] For *Mycobacterium fortuitum*, the second most frequent RGM, a combination of two or three drugs is recommended and should include a fluoroquinolone, co-trimoxazole, amikacin, linezolid, imipenem or tetracycline, again based on *in vitro* susceptibility. However, there is limited clinical evidence to support these treatment regimens.[4] Even at the more fundamental level, little

is known about the pharmacodynamics of commonly used antibiotics against RGM.

Time–kill assays allow the study of the pharmacodynamics of antibiotics, examining the rate at which different concentrations of an antibiotic kill bacteria; the concentration-dependent and time-dependent bactericidal activities of antimicrobial agents such as aminoglycosides, fluoroquinolones or β-lactams and macrolides can be studied using this methodology. The purpose of this study was to assess the pharmacodynamics of commonly used drugs to treat RGM disease by means of the time–kill assay and subsequent modelling of the results to assist in a more rational design of treatment regimens.

Materials and methods

Bacterial strains

We used *M. abscessus* subspecies *abscessus* CIP 104536 (Collection of Institute Pasteur, Paris, France) and *M. fortuitum* ATCC 6841 (ATCC, Rockville, MD, USA) for the experiments. Stock vials of each mycobacterium were preserved at −80°C in trypticase soy broth with 40% glycerol and were thawed for each assay.

Antibiotics

Moxifloxacin, cefoxitin, amikacin and clarithromycin were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands) and linezolid was obtained from Pfizer BV (Capelle aan den Ijssel, the Netherlands) as the 2 mg/mL infusion. Water was the solvent for preparing stock solutions, except for clarithromycin, which was dissolved in methanol. Stock solutions were stored at −80°C; prior to each experiment, one aliquot was thawed to prepare the different concentrations to be tested.

Susceptibility testing

The MIC of each of the tested antibiotics was determined by broth microdilution in cation-adjusted Mueller–Hinton broth (CAMHB; BD Bioscience, Erembodegem, Belgium) at 30°C, according to CLSI guidelines.[5] For the initial evaluation, commercial panels were used (RAPMYCO Sensititre®, Trek Diagnostics/ThermoFisher, Landsmeer, the Netherlands) following the manufacturer's recommendations. If the MIC fell outside the concentration range tested in the commercial assay, we performed manual broth microdilution with wider concentration ranges.

Time–kill assays

The mycobacterial inoculum was prepared freshly for each experiment by growing over 24 h in CAMHB with oleic acid/bovine albumin/dextrose/catalase (OADC) growth supplement (BD Bioscience, Erembodegem, Belgium) and 0.05% Tween 80 (Sigma-Aldrich), to obtain bacteria in the early logarithmic phase of growth. Individual bottles of 20 mL of CAMHB plus OADC and 0.05% Tween 80 containing eight 2-fold increasing concentrations of each antibiotic (from 0.25 to 32 times the MIC, except for *M. abscessus* and cefoxitin, for which two lower concentrations were included) were cultured with the inoculum (density ~10^5–10^6 cfu/mL) at 30°C, under shaking conditions (100 rpm); ventilation through a bacterial filter (FP 30/0.2 Ca/S, Whatman GmbH, Germany) was incorporated. A growth control bottle, with inoculum but without antibiotic, as well as a sterility control (medium only) were included. At defined time intervals (3, 6, 12, 24, 36, 48, 72, 96 and 120 h), the size of the bacterial population was quantified to characterize the effect of the different antibiotics. Samples of 1 mL were taken from each bottle and serial 10-fold dilutions in 0.85% sterile saline solution were prepared. Volumes of 10 μL from undiluted samples and from each dilution were plated in triplicate on Middlebrook 7H11 (BD Bioscience, Erembodegem, Belgium) for further cfu counting after 3–5 days of incubation at 30°C. The theoretical detection limit was 33.3 cfu per plate, corresponding to 1.52 $\log_{10}$ cfu/mL.

Curve fitting and analysis

The experimental data derived from time–kill assays were analysed using GraphPad Prism 5.03 (GraphPad Inc., San Diego, CA, USA). Log cfu values were plotted against time for each antibiotic. The kill rate was determined at different time intervals (3–24, 3–36, 24–72, 24–96 and 24–120 h), undertaking a linear regression to find the slope for each concentration; the logarithmic transformed concentration was then plotted against each slope and a non-linear regression analysis (dose–response) was run. The sigmoid maximum effect (E_{max}) model (four-parameters Hill's equation)[6,7] was fitted to the kill rate data, analysing each assay to determine the pharmacodynamic relationship between the antibiotic concentration and bacterial growth or death. E_{max}, 50% effective concentration (EC_{50}), Hill's slope (γ) and R^2 were calculated for each assay.

Results

Susceptibility

The MICs determined for each antibiotic are shown in Table 1. All the MICs were higher for *M. abscessus* than for *M. fortuitum*.

Time–kill assays

Figures 1 and 2 show the pattern of growth and kill by antibiotics of *M. abscessus* and *M. fortuitum*, respectively, at different concentrations of each of the tested antibiotics. The growth curves differ for each species. *M. abscessus* showed a lag phase of 3–12 h and its maximum growth was higher than the growth for *M. fortuitum* in all the experiments. The lag phase for *M. fortuitum* was around 3 h. In general, time–kill curves for *M. abscessus* showed smaller decreases in bacterial population size than those observed for *M. fortuitum* when exposed to antibiotics.

For *M. abscessus*, the cefoxitin time–kill curve was different from those of amikacin and clarithromycin. After a short lag

Table 1. Susceptibility data for *M. abscessus* and *M. fortuitum* type strains tested by broth microdilution in CAMHB, reading at 72 h[5]

	MIC (mg/L)				
Strain	cefoxitin	amikacin	moxifloxacin	linezolid	clarithromycin
M. abscessus CIP 104536	64	32	8	32	4
M. fortuitum ATCC 6841	32	1	0.062	8	2

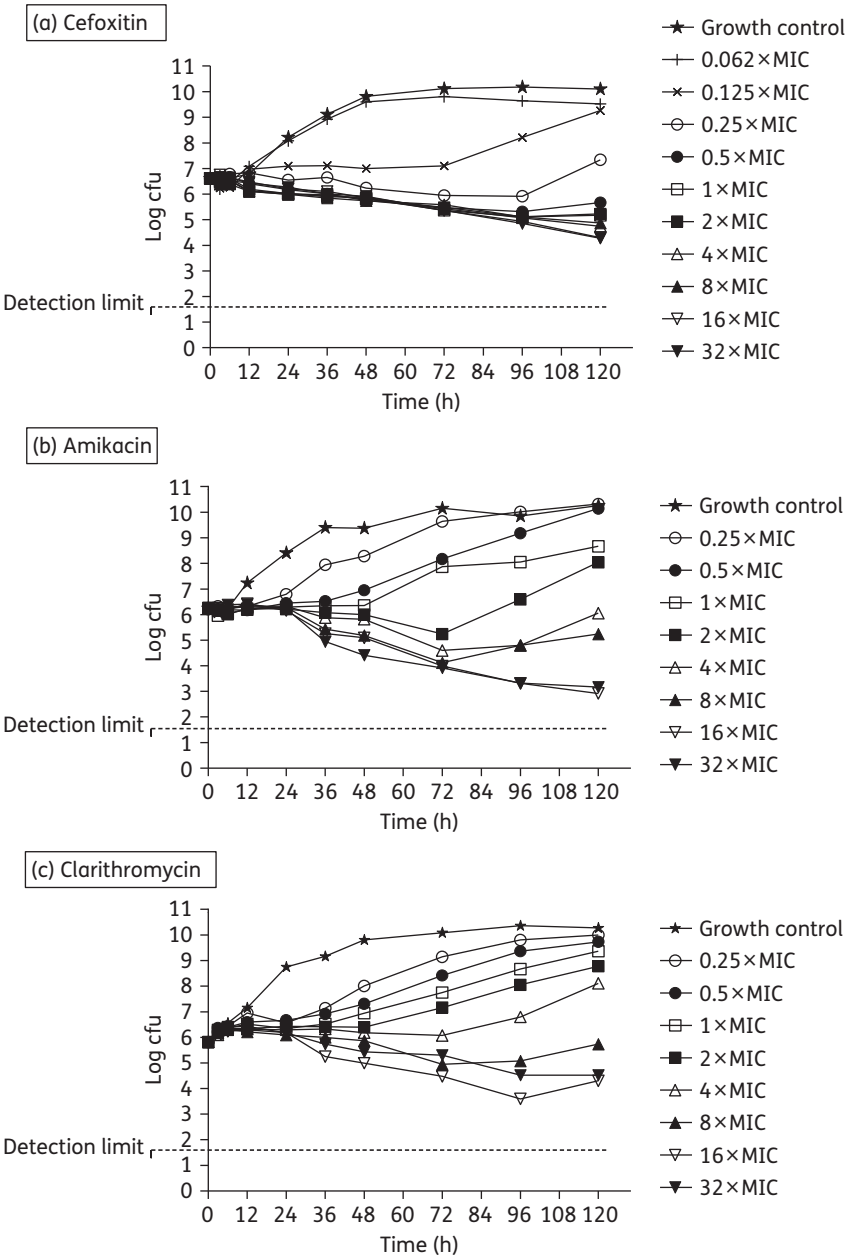

Figure 1. Time–kill curves of (a) cefoxitin, (b) amikacin and (c) clarithromycin against *M. abscessus* CIP 104536. Antibiotic concentrations are indicated by different symbols.

phase, a slow decline was observed at almost all concentrations, reaching the lowest cfu counts only after 96–120 h of incubation. In contrast, during amikacin and clarithromycin exposure, killing was observed after only 24 h and for some concentrations, in particular with clarithromycin, there appeared to be significant growth even before killing was observed. Only after 24 h did concentrations higher than 2×MIC started to effectively decrease the bacterial density, with its maximum decrease at 120 h at the two highest concentrations. Regrowth was, however, observed with 2, 4 and 8×MIC.

Interestingly, after 48 h, part of the colonies exposed to amikacin concentrations of 2×MIC and higher converted to a rough morphology, which was observed after plating the samples for cfu counting.

Significant differences in the killing characteristics of the antibiotics were observed for *M. fortuitum*. Linezolid showed only slight but prolonged killing without apparent concentration-dependent effects. The curves for cefoxitin, amikacin and moxifloxacin shared some characteristics and exhibited an important reduction in growth during the first 24–36 h (Figure 2); however, regrowth occurred after the initial killing and was noticeable for amikacin in particular, with the phenomenon also observed for *M. abscessus*. Amikacin also showed the highest kill rate and concentration-dependent activity; the main fall in cfu was

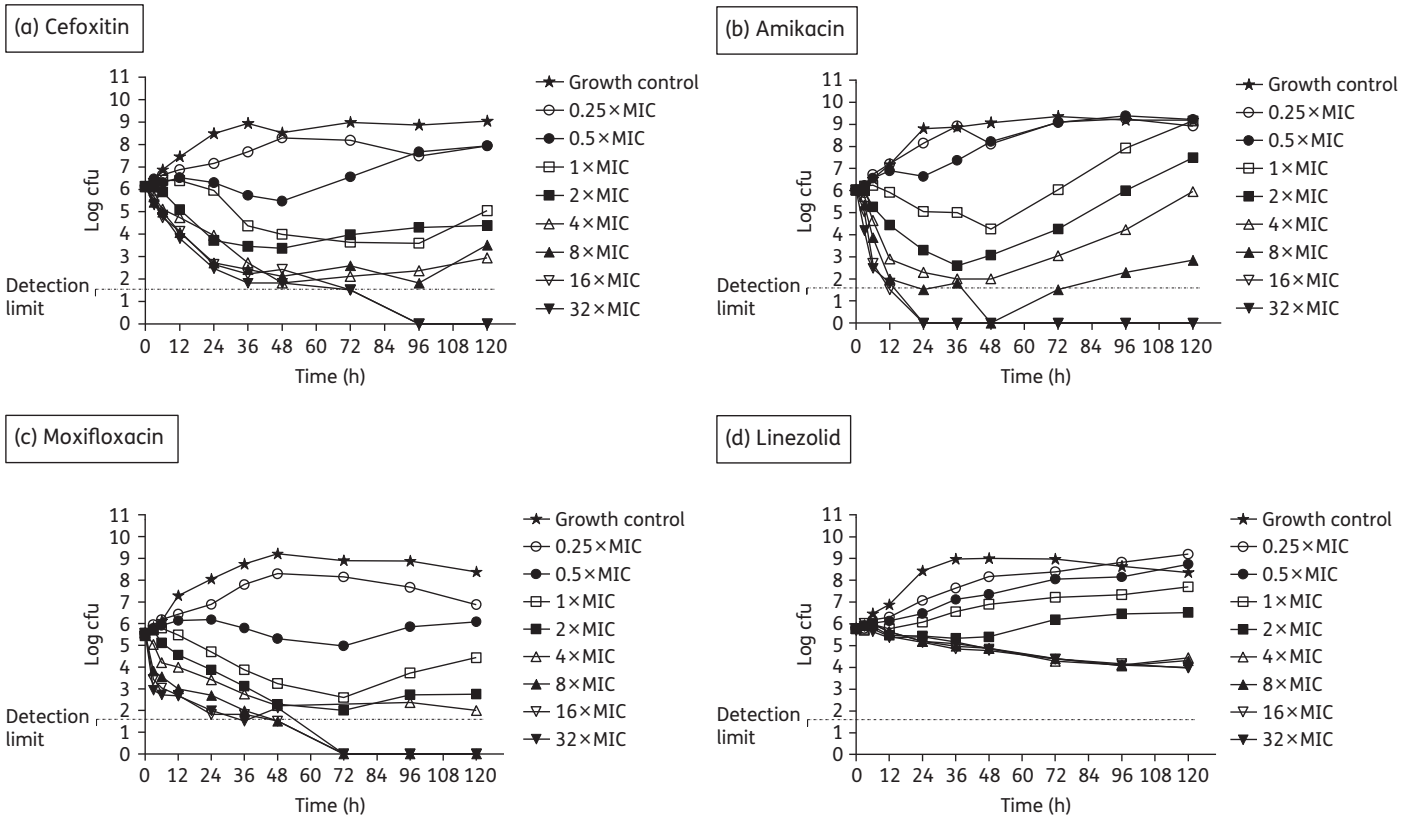

Figure 2. Time–kill curves of (a) cefoxitin, (b) amikacin, (c) moxifloxacin and (d) linezolid against *M. fortuitum* ATCC 6841. Antibiotic concentrations are indicated by different symbols.

observed at concentrations 4×MIC or higher. The same phenomenon of morphology changes noted previously for *M. abscessus* was observed for *M. fortuitum* colonies exposed to 2×MIC or higher of amikacin.

Time–kill modelling

After a lag phase, the maximum kill rate was present over the period 24–72 h for *M. abscessus* and 3–24 h for *M. fortuitum*. Figure 3 shows the relationship between the kill rate and concentration for the two RGM species. Pharmacodynamic parameter estimates were obtained with the E_{max} model with a variable slope (Table 2). The E_{max} model fitted well and confirmed that the kill rate for *M. abscessus* was relatively low. The highest killing rate was observed for amikacin, 0.0427 h^{-1}, between 24 and 72 h. There was no significant difference in Hill's slope estimated for the antibiotics tested ($P=0.2213$), indicating that the differences in effect modality are primarily determined by the maximum kill rate of each antibiotic.

For *M. fortuitum*, *the* maximum kill rate was observed much earlier, between 3 and 24 h of exposure, and again amikacin showed the highest E_{max}, 0.1933 h^{-1}. As with *M. abscessus*, no significant differences were found between the Hill's slope calculated for each of the antibiotics tested ($P=0.2696$), indicating that the effect is primarily determined by the maximum effect.

Discussion

This study provides fundamental new information on the pharmacodynamic relationship between antibiotic concentrations and mycobacterial population dynamics for RGM.

Amikacin, cefoxitin and clarithromycin did not show a high killing effect on *M. abscessus*, although killing proceeded with time. For *M. fortuitum*, amikacin, cefoxitin and moxifloxacin showed highest killing rates. The higher growth level consistently reached by *M. abscessus* could, at least in part, explain the lesser effect observed for the antibiotics on this species.

The individual analysis of each antibiotic indicated that amikacin had the highest effect on both RGM. For *M. abscessus*, amikacin inhibited the growth during the first 24 h, but the killing activity started after that time. For *M. fortuitum*, killing from amikacin started earlier. We observed morphological changes after exposure to amikacin concentrations of 2×MIC or higher. This phenomenon has been previously observed for *Pseudomonas aeruginosa* after 6 h of incubation in time–kill assays.[8] Whether the changes we observed are related to the appearance of resistant mutants or are the result of an adaptation response should be addressed in the future.

Recently published work with clinical strains of *M. abscessus* exposed to amikacin, as well as to linezolid, tigecycline and moxifloxacin, states a lack of antimicrobial bactericidal activity of these antibiotics.[9] However, the assays were conducted only until 24 h,

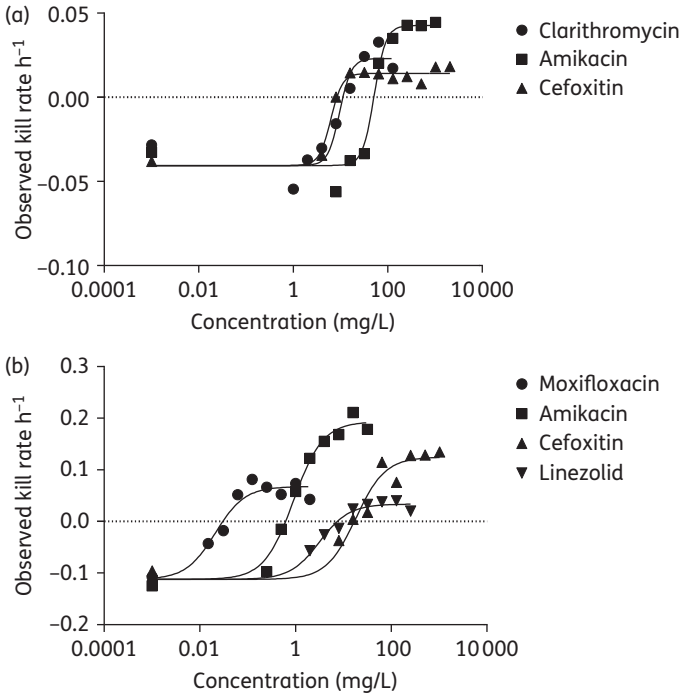

Figure 3. The best-fit sigmoid curves obtained from the E_{max} model of (a) *M. abscessus* CIP 104536 exposed to cefoxitin, amikacin and clarithromycin, between 24 and 72 h, and (b) *M. fortuitum* ATCC 6841 exposed to cefoxitin, amikacin, moxifloxacin and linezolid, between 3 and 24 h. A different *y*-axis scale is used for (a) and (b).

a difference from the present study, which describes the activity over 120 h assays; the observed lack of activity by the authors may therefore be largely explained by the lag phase we have also observed. Our results clearly indicate that the effects of antimicrobials on *M. abscessus* should be studied over at least 72 h to provide useful information.

Although it involved a different NTM, previous work on *Mycobacterium avium* complex (MAC) showed that amikacin appeared highly and rapidly bactericidal in the early logarithmic phase of growth.[10] Moreover, with *Mycobacterium tuberculosis*, the time–kill kinetics of amikacin displayed a high and extremely rapid killing activity that was not time dependent and could eliminate all mycobacteria.[11] Against *M. abscessus* and *M. fortuitum*, we observed a significantly weaker effect. Nonetheless, amikacin still can play an important role in the treatment of disease caused by RGM.

The effect of cefoxitin was different in the two RGM species evaluated. For *M. abscessus*, cefoxitin behaved like other β-lactams and the bacterial density gradually declined during exposure to it. This is in contrast to the effect observed in *M. fortuitum*, where cefoxitin showed a concentration-dependent effect and the second highest E_{max}. Differences in the effect of cefoxitin have previously been reported in MRSA. Combining cefoxitin with a variety of β-lactams enhanced their *in vitro* activity against community-acquired MRSA strains but not against hospital-acquired MRSA; this may result from the differential binding of cefoxitin to the target (penicillin-binding protein 4, PBP4), which plays an important role only in the β-lactam resistance of community-acquired strains.[12] Cefoxitin targets or mechanisms of action may not be the same

Table 2. Parameter estimates, with 95% CI, derived from the E_{max} model fitted to time–kill assay data

Strain	Antibiotic	Time (h)	E_{max} (h^{-1})	95% CI	EC_{50} (mg/L)	95% CI	Hill's slope (γ)[a]	95% CI	R^2
M. abscessus CIP 104536	cefoxitin	24–72	0.0142	0.008719–0.01976	6.24	4.450–8.735	3.534	1.804–5.263	0.97
	amikacin	24–72	0.0427	0.03456–0.05083	51.74	41.18–65.01			0.97
	clarithromycin	24–72	0.0231	0.01430–0.03185	9.63	7,163–12.96			0.92
M. fortuitum ATCC 6841	cefoxitin	3–24	0.1247	0.1041–0.1454	18.24	12.78–26.02	1.416	1.021–1.811	0.93
	amikacin	3–24	0.1933	0.1689–0.2177	0.910	0.6848–1.209			0.99
	moxifloxacin	3–24	0.0677	0.04939–0.08590	0.021	0.01295–0.03445			0.93
	linezolid	3–24	0.0338	0.01518–0.05234	3.203	1.784–5.750			0.97

[a]Pooled value for all antibiotics by species.

in *M. abscessus* and *M. fortuitum*; this could explain the differences observed and will be an interesting subject for further evaluation.

Clarithromycin, long considered a cornerstone of *M. abscessus* treatment,[1,3] showed killing capacity only at concentrations greater than 4×MIC. Similarly, previous studies of MAC observed a maximum bactericidal effect at a relatively high concentration (256 mg/L[10]). Linezolid and moxifloxacin were only tested for *M. fortuitum* as these drugs were considered inactive against the *M. abscessus* type strain (Table 1); however, their killing capacity was not high for *M. fortuitum.* Few comparative data are available for those antibiotics.

According to the E_{max} model fitted to our data, Hill's slopes were not significantly different between the antibiotics tested for each species. In this regard, the total effect observed was primarily determined by the E_{max}, representing the extent of kill as a function of concentration, and not by the Hill's slope. This is different from the effect of antibiotics in other bacterial species, where a clear difference is observed for time-dependent antimicrobials such as β-lactams (lower E_{max}, higher Hill slope) and concentration-dependent drugs such as aminoglycosides (higher E_{max}, lower Hill slope).[7]

Our data for *M. abscessus* contrast with the recent findings from the nude mice model, in which cefoxitin was superior in efficacy to amikacin and clarithromycin. Only cefoxitin improved survival and reduced bacillary loads in the spleen; amikacin and clarithromycin prevented death but had little impact on bacillary loads. Interestingly, in this model the amikacin/clarithromycin/cefoxitin combination was as active as cefoxitin alone.[13]

Extrapolating these data to the treatment setting, the limited activity detected for amikacin, cefoxitin and clarithromycin against *M. abscessus* fits well with the clinical observations that treatment with regimens containing these drugs leads to poor outcomes.[3] The amikacin peak serum concentration in patients with NTM pulmonary disease averages 55 mg/L,[14,15] i.e. around the MIC measured for the *M. abscessus* type strain. Given that the activity of amikacin was best at the highest concentrations, the current dosing may not yield concentrations at the site of infection that can exhibit significant killing activity. This may in part explain the limited efficacy of amikacin against *M. abscessus* in the nude mice model.[13] Local administration, e.g. inhaled amikacin for pulmonary disease caused by *M. abscessus*, may be more efficacious. The *M. abscessus* type strain had an MIC of clarithromycin of 4 mg/L, and the maximum effect was attained at concentrations >2×MIC. These concentrations are above the concentration attainable in the serum of patients (which average 4 mg/L[4,14,15]), but the macrolides are known to accumulate in lung tissue, epithelial lining fluid and macrophages at concentrations 2–200 times higher than serum concentrations,[16] although it is not clear whether these concentrations are active.[17] Hence, the high concentrations needed to achieve a significant effect in our time–kill assay may be attainable at the site of infection in *M. abscessus* lung disease. Unfortunately, no pharmacokinetic data are available for cefoxitin in this patient category, which needs to be investigated if cefoxitin is to continue to be included in the treatment regimens for RGM disease.

The time–kill assays performed in this study provide basic information on the individual effect of static concentrations of each antibiotic on *M. abscessus* and *M. fortuitum*. However, this setting is very different from the *in vivo* situation where multidrug therapy, the daily intake of drugs and their pharmacokinetics, and the localization of the causative mycobacteria create a very different scenario. Thus, the next step should be to continue these studies in dynamic pharmacokinetic/pharmacodynamic models for the evaluation of combined regimens (which are, and should be, the norm) in RGM disease. These models, despite their abstractions, will provide data that are much closer to the *in vivo* situation and may aid in the design of more active treatment regimens.

In conclusion, time–kill kinetic assays revealed that amikacin was more active than clarithromycin and cefoxitin against *M. abscessus* and that amikacin, followed by cefoxitin, moxifloxacin and linezolid, was also most active against *M. fortuitum*. However, we demonstrate that the activity of all the drugs tested was relatively low and the concentrations effective *in vitro* can hardly be reached *in vivo*, reminiscent of the poor outcomes of antibiotic treatment for RGM diseases.

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Transparency declarations

None to declare.

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