Inhibition of the β-Lactamase Bla Mab by Avibactam Improves the In Vitro and In Vivo Efficacy of Imipenem against Mycobacterium abscessus

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Inhibition of the β-Lactamase Bla_{Mab} by Avibactam Improves the *In Vitro* and *In Vivo* Efficacy of Imipenem against *Mycobacterium abscessus*

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ABSTRACT *Mycobacterium abscessus* pulmonary infections are treated with a macrolide (clarithromycin or azithromycin), an aminoglycoside (amikacin), and a β-lactam (cefoxitin or imipenem). The triple combination is used without any β-lactamase inhibitor, even though *M. abscessus* produces the broad-spectrum β-lactamase Bla_{Mab}. We determine whether inhibition of Bla_{Mab} by avibactam improves the activity of imipenem against *M. abscessus*. The bactericidal activity of drug combinations was assayed in broth and in human macrophages. The *in vivo* efficacy of the drugs was tested by monitoring the survival of infected zebrafish embryos. The level of Bla_{Mab} production in broth and in macrophages was compared by quantitative reverse transcription-PCR and Western blotting. The triple combination of imipenem (8 or 32 μg/ml), amikacin (32 μg/ml), and avibactam (4 μg/ml) was bactericidal in broth (<0.1% survival), with 3.2- and 4.3-$\log_{10}$ reductions in the number of CFU being achieved at 72 h when imipenem was used at 8 and 32 μg/ml, respectively. The triple combination achieved significant intracellular killing, with the bacterial survival rates being 54% and 7% with the low (8 μg/ml) and high (32 μg/ml) dosages of imipenem, respectively. *In vivo* inhibition of Bla_{Mab} by avibactam improved the survival of zebrafish embryos treated with imipenem. Expression of the gene encoding Bla_{Mab} was induced (20-fold) in the infected macrophages. Inhibition of Bla_{Mab} by avibactam improved the efficacy of imipenem against *M. abscessus in vitro*, in macrophages, and in zebrafish embryos, indicating that this β-lactamase inhibitor should be clinically evaluated. The *in vitro* evaluation of imipenem may underestimate the impact of Bla_{Mab}, since the production of the β-lactamase is inducible in macrophages.

KEYWORDS β-lactamase inhibitor, avibactam, *Mycobacterium abscessus*, cystic fibrosis, imipenem

In the context of cystic fibrosis, *Mycobacterium abscessus* has emerged in recent years as an important opportunistic lung pathogen increasingly responsible for mortality (1–5). These infections are extremely difficult to treat and to eradicate, as *M. abscessus* is naturally resistant to most antibiotics, including antituberculous agents (6, 7). The recommended treatment for pulmonary infections relies on the combination of a macrolide (clarithromycin or azithromycin), an aminoglycoside (amikacin), and an intravenous β-lactam (cefoxitin or imipenem) for at least 12 to 16 months (6, 8). Resistance to macrolide, present in 40 to 60% of the isolates (9), leads to a cure rate of only 25 to 40% (whereas the

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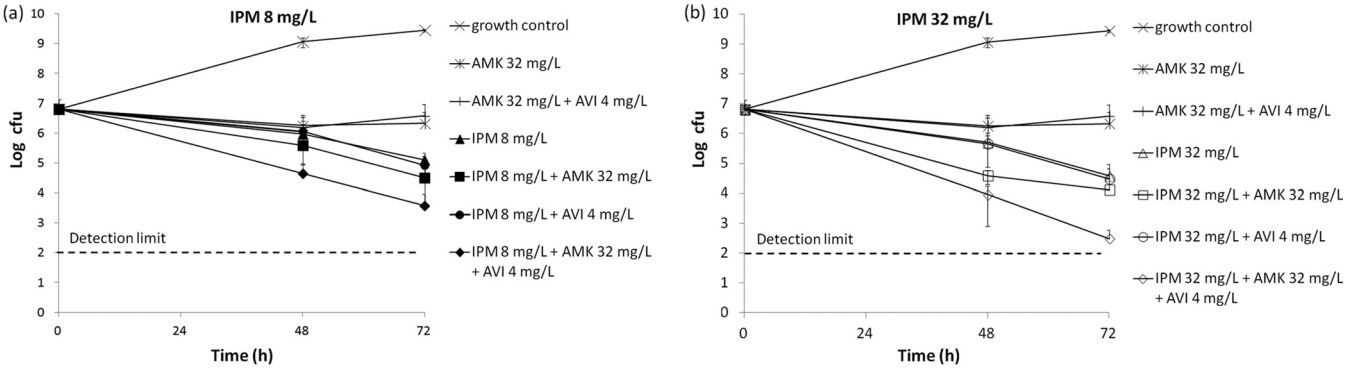

FIG 1 Time-kill curves of the activity of imipenem (IPM) alone or in combination with the β-lactamase inhibitor avibactam (AVI) and amikacin (AMK) against *M. abscessus* CIP104536 S. (a) Time-kill curves of imipenem at 8 μg/ml alone or in combination with avibactam (4 μg/ml) and amikacin (32 μg/ml). The number of CFU was determined after 0, 48, and 72 h of exposure to the antibiotics. (b) Time-kill curves of imipenem at 32 μg/ml alone or in combination with avibactam (4 μg/ml) and amikacin (32 μg/ml).

cure rate is 88 to 95% for patients infected with macrolide-susceptible isolates). Cefoxitin and imipenem are the most active β-lactams, in spite of moderate *in vitro* activity with MICs (MIC_{50}s) of 32 and 16 μg/ml, respectively (10).

We have previously shown that the production of a broad-spectrum β-lactamase, the *M. abscessus* β-lactamase (Bla_{Mab}), is a major determinant of β-lactam resistance in *M. abscessus* (11, 12). In contrast to the β-lactamase BlaC from *M. tuberculosis*, Bla_{Mab} is not inactivated by clavulanate, sulbactam, and tazobactam, since these inhibitors are hydrolyzed by the β-lactamase. However, Bla_{Mab} is efficiently inhibited by avibactam (12), a β-lactamase inhibitor approved by the FDA in 2014 (13, 14). Deletion of the gene encoding Bla_{Mab} or chemical inhibition of the β-lactamase activity by avibactam extends the spectrum of β-lactams active against *M. abscessus*, as previously shown for an extensive evaluation of the amoxicillin-avibactam combination (12).

Recently, we have reported that the rate of killing of an *M. abscessus* Bla_{Mab}-deficient mutant by the combination of imipenem and amikacin is significantly higher than that of the parental strain (15). In macrophages, the difference was even more pronounced, since a 100-fold reduction in the number of intracellular bacteria was observed for the Bla_{Mab}-deficient mutant, whereas the combination was only bacteriostatic for the wild-type strain (15). Together, the results obtained with the Bla_{Mab}-deficient mutant indicated that the production of the β-lactamase may limit the efficacy of imipenem, despite the fact that this drug is used in the absence of a β-lactamase inhibitor in the recommended treatment of pulmonary infections due to *M. abscessus*. In this study, we evaluated whether Bla_{Mab} inhibition by avibactam could potentiate the effects of imipenem *in vitro*, in macrophages, and in zebrafish embryos. The zebrafish embryo model has been developed to assess the *in vivo* activity of antibiotics against *M. abscessus* (16). Unexpectedly, we found that the production of Bla_{Mab} has a greater impact on the activity of imipenem in macrophages than in *in vitro* cultures, prompting us to determine and compare the level of Bla_{Mab} production in planktonically and intracellularly growing *M. abscessus* bacteria.

RESULTS

***In vitro* killing of *M. abscessus* CIP104536 S by imipenem alone or in combination with amikacin and avibactam.** Imipenem was tested at 8 μg/ml and 32 μg/ml, doses that correspond to concentrations equal to 4- and 16-fold the MICs of the drug against *M. abscessus* CIP104536 with a smooth morphotype (CIP104536 S), respectively (15). Amikacin was tested at 4-fold the MIC (32 μg/ml). Avibactam was tested at 4 μg/ml, which is the concentration used for susceptibility testing in *Enterobacteriaceae* (17, 18). Reductions in the $\log_{10}$ number of CFU of *M. abscessus* CIP104536 S were observed for imipenem at 8 μg/ml when it was tested alone (1.7 $\log_{10}$) or in combination with amikacin (2.3 $\log_{10}$) or avibactam (1.9 $\log_{10}$) (Fig. 1a; see Table S2 in the

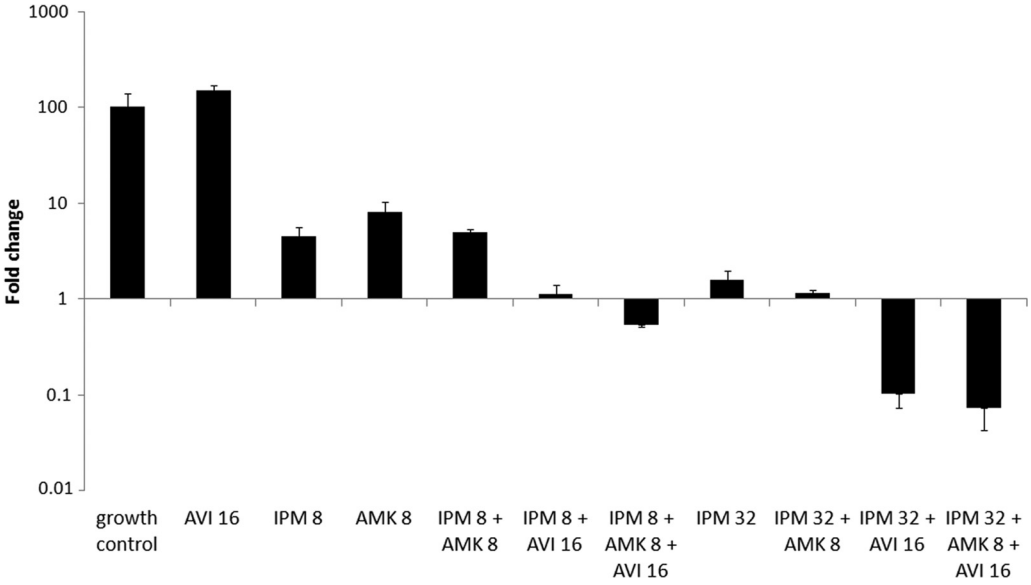

FIG 2 Intracellular activity of imipenem (IPM; 8 μg/ml and 32 μg/ml) alone or in combination with the β-lactamase inhibitor avibactam (AVI; 16 μg/ml) and amikacin (AMK; 8 μg/ml) against *M. abscessus* CIP104536 S. Intracellular bacteria were enumerated, and the fold change in the number of CFU was determined at between days 0 and 2 postinfection. Bars represent standard deviations.

supplemental material for the results of statistical analysis). The triple combination of imipenem-amikacin-avibactam was bactericidal (less than 0.1% survival), achieving a 3.2-$\log_{10}$ reduction in the number of CFU. The triple combination was more active than imipenem plus avibactam (3.2- versus 1.9-$\log_{10}$ reductions; $P < 0.05$), but the difference from the results obtained with imipenem plus amikacin was not significant (3.2- versus 2.3-$\log_{10}$ reductions; $P = 0.12$). Increasing the imipenem concentration from 8 μg/ml (Fig. 1a) to 32 μg/ml (Fig. 1b) moderately increased the activity of imipenem alone (1.7- versus 2.2-$\log_{10}$ reductions), of imipenem combined with amikacin (2.3- versus 2.7-$\log_{10}$ reductions), and of imipenem combined with avibactam (1.9- versus 2.3-$\log_{10}$ reductions), but none of these differences were statistically significant. Imipenem at 32 μg/ml combined with amikacin and avibactam was the most active drug combination, achieving a 4.3-$\log_{10}$ reduction.

Intramacrophage activity of imipenem alone or in combination with amikacin and avibactam. THP-1-derived macrophages were infected with *M. abscessus* CIP104536 S and exposed to various drugs for 2 days, and the surviving bacteria were enumerated by plating serial dilutions of macrophage lysates (Fig. 2; see Table S3 in the supplemental material for the results of statistical analysis). In the absence of antibiotic, *M. abscessus* CIP104536 S grew in macrophages, leading to a 100-fold increase in the number of CFU at 2 days. Imipenem at 8 μg/ml (Fig. 2) partially prevented the intramacrophage growth of *M. abscessus* CIP104536 S (4.5- versus 100-fold increases in the number of CFU; $P < 0.05$). Amikacin was also active (8-fold increase in the number of CFU; $P < 0.05$). The combination of imipenem and amikacin was not more active than imipenem alone (4.5- versus 5.0-fold increases in the number of CFU, respectively; $P = 0.51$). In contrast, avibactam improved the activity of imipenem, preventing the intracellular proliferation of *M. abscessus* CIP104536 S (1.1- versus 4.5-fold increases in the number of CFU; $P < 0.05$). The combination of imipenem (8 μg/ml), amikacin, and avibactam was the only combination that reduced the number of intracellular bacteria (fold change in the number of CFU, 0.54). This value was significantly different from that obtained with imipenem combined with avibactam (fold change in the number of CFU, 0.54 versus 1.1; $P < 0.05$). Increasing the concentration of imipenem from 8 μg/ml to 32 μg/ml improved the activity of the drug tested alone (4.5- versus 1.6-fold change in the number of CFU) and in combination with amikacin (5.0- versus 1.1-fold change),

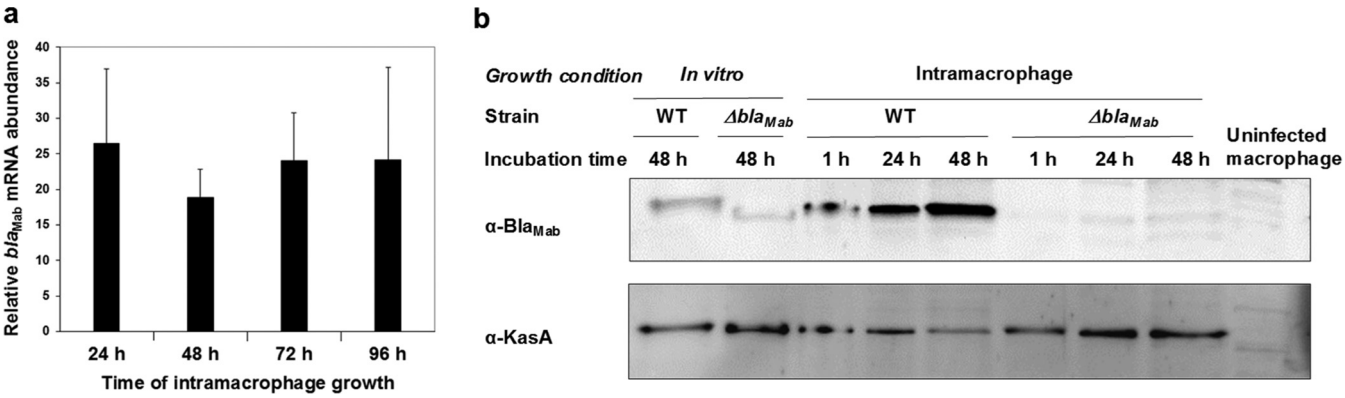

FIG 3 Production of Bla_{Mab} *in vitro* and in macrophages. (a) Quantification of bla_{Mab} by qRT-PCR. RNA was isolated from wild-type strain CIP CIP104536 S grown *in vitro* in 7H9 medium for 48 h or in human J774 macrophages for 24 h, 48 h, 72 h, and 96 h. *sigA* rRNA was used as an internal standard. The values are the ratio of intramacrophage growth to *in vitro* growth. Results are expressed as the means ± standard deviations from three experiments performed in triplicate. Bars represent standard deviations. (b) Immunodetection of Bla_{Mab}. Protein extracts were prepared from wild-type strain CIP104536 S and its Δbla_{Mab} derivative grown *in vitro* in 7H9 medium for 48 h or in human J774 macrophages for 1 h, 24 h, and 48 h. Proteins (3 μg) were separated by 15% SDS-PAGE. Immunodetection was performed with a mouse immune serum specific for Bla_{Mab} and a peroxidase-conjugated goat anti-mouse immunoglobulin antibody. Immunodetection of KasA was used as a loading control.

avibactam (1.1- versus 0.1-fold change), and amikacin and avibactam (0.54- versus 0.07-fold change). The latter differences were significant ($P < 0.05$). In conclusion, avibactam significantly improved the activity of imipenem when it was tested both with and without amikacin. Significant intracellular killing was obtained with the double and triple combinations involving imipenem at 32 μg/ml and avibactam with or without amikacin.

Bla_{Mab} is produced at a high level within macrophages. The expression of bla_{Mab} was investigated at both the transcriptional and translational levels in *M. abscessus* bacteria grown *in vitro* or proliferating in infected macrophages. Quantitative reverse transcription-PCR (qRT-PCR) analyses indicated that the growth of *M. abscessus* within macrophages led to a 20-fold increase in the relative abundance of the bla_{Mab} transcript in comparison to that in planktonic cultures performed *in vitro* in 7H9sB medium (Fig. 3a). Western blot analysis confirmed that Bla_{Mab} is produced at a higher level in macrophages (Fig. 3b).

Avibactam increases the efficacy of imipenem in the zebrafish model of *M. abscessus* infection. The zebrafish model of *M. abscessus* infection (16) was used to assess the *in vivo* efficacy of imipenem following inhibition of Bla_{Mab} by avibactam (Fig. 4). Although imipenem alone was active, avibactam further increased larval survival ($P < 0.05$ for both concentrations of imipenem tested). These results indicate that the production of Bla_{Mab} during infection impairs the efficacy of imipenem and that chemical inhibition by avibactam overcomes the deleterious effect of the β-lactamase.

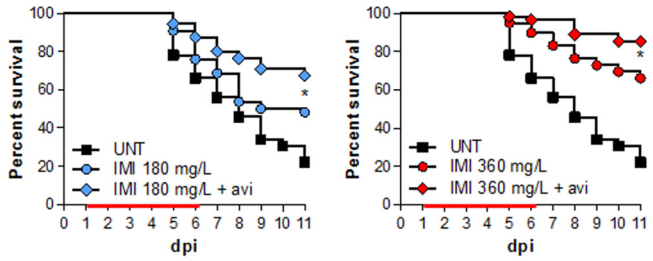

FIG 4 Efficacy of imipenem alone or in combination with avibactam in zebrafish embryos infected by *M. abscessus* CIP104536 R expressing TdTomato. Embryos (60/300 per group) were infected at 30 h postfertilization and exposed to imipenem (IMI) from 1 to 6 days postinfection (dpi) at two concentrations (180 [left] and 360 μg/ml [right]) alone or in combination with avibactam at 50 μg/ml (avi). Control animals were left untreated (UNT). The survival of animals was monitored each day postinfection, and the results were expressed as the percent larval survival. *, $P < 0.05$.

DISCUSSION

Here, we assessed whether the inhibition of the β-lactamase Bla_{Mab} by avibactam improves the efficacy of imipenem against *M. abscessus*. The impact of the β-lactamase inhibitor was assessed *in vitro* and in infected macrophages by determining the killing activity of various drug combinations. Avibactam was also investigated by monitoring the survival of infected zebrafish embryos treated with imipenem alone or in combination with the inhibitor. In macrophages, avibactam significantly improved the activity of imipenem in all drug combinations tested, which included two dosages of imipenem with or without amikacin (Fig. 2). Imipenem combined with avibactam and amikacin was highly active in macrophages, leading to 93% intracellular killing at the highest dose tested. In the zebrafish model, imipenem alone increased the survival of the embryos, as previously reported (16), and the efficacy of the drug was significantly improved by avibactam (Fig. 4). Amikacin could not be tested in this model due to its toxicity during larval development (data not shown). Time-kill curves showed that the triple combinations of imipenem (8 and 32 μg/ml), avibactam, and amikacin were bactericidal (less than 0.1% survival; Fig. 1a and b). These associations were the most active, although statistical analysis did not demonstrate a significant impact of avibactam. The bla_{Mab} transcript was 20-fold less abundant in *M. abscessus* bacteria proliferating in planktonic cultures than in *M. abscessus* bacteria in infected macrophages (Fig. 3). Thus, the high level of production of Bla_{Mab} in the infected macrophages, which was confirmed by Western blotting, may account for the fact that avibactam has a greater impact on the efficacy of imipenem intracellularly than *in vitro*. The induction of bla_{Mab} in macrophages indicates that the *in vitro* evaluation of imipenem may underestimate the impact of the β-lactamase Bla_{Mab}. Of note, there is only a 2-fold difference in the MIC of imipenem against the Bla_{Mab}-deficient mutant of *M. abscessus* and that against the parental strain (19).

Two β-lactams, cefoxitin and imipenem, are recommended for the treatment of pulmonary infections due to *M. abscessus* (8). Currently, there is no recommendation for the preferential use of one these drugs. We have previously shown that Bla_{Mab} hydrolyzes imipenem with a moderate but significant catalytic efficacy (3×10^4 M^{-1} s^{-1}) (11). Bla_{Mab} is 4,000-fold less active for the hydrolysis of cefoxitin. The low efficacy of hydrolysis of cefoxitin by Bla_{Mab} is likely to be irrelevant since the killing curve assay did not reveal any difference in the activity of cefoxitin, alone or in combination with amikacin, against a Bla_{Mab}-deficient mutant of *M. abscessus* and the parental strain (15). In this study, we have shown that imipenem is highly active in macrophages, but this requires combination with avibactam due to the induction of bla_{Mab}. Together, these data indicate that imipenem is intrinsically more active than cefoxitin, although this difference is compensated for by the higher level of hydrolysis of imipenem by the β-lactamase Bla_{Mab}.

The assessments of the efficacy of drug combinations *in vitro* (Fig. 1), in macrophages (Fig. 2), and in the zebrafish (Fig. 4) indicate that the triple combination of avibactam, imipenem, and amikacin should be clinically evaluated, particularly in infections due to clarithromycin-resistant *M. abscessus*, which are often not cured by the recommended treatments (7, 20–22). Unfortunately, avibactam is currently manufactured in combination with ceftazidime, which has no activity against *M. abscessus* (12). Formulation of avibactam independently from any β-lactam partner would be necessary to provide cystic fibrosis patients access to the avibactam-imipenem-amikacin combination.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. abscessus* CIP104536 (ATCC 19977) with a smooth (S) or rough (R) morphotype and the respective β-lactamase-deficient derivatives (Δbla_{Mab}) (12) were grown in Middlebrook 7H9 broth (BD-Difco, Le Pont de Claix, France) supplemented with 10% (vol/vol) oleic acid, albumin, dextrose, and catalase (OADC; BD-Difco) and 0.05% (vol/vol) Tween 80 (Sigma-Aldrich) (7H9sB) at 30°C with shaking (150 rpm) (23).

Antibiotics. Amikacin was provided by Bristol-Myers Squibb (Rueil-Malmaison, France), and imipenem was provided by Mylan (Saint-Priest, France). Avibactam was provided by AstraZeneca. Water was the solvent used to prepare stock solutions, which were freshly prepared for each experiment and filtered

using a sterilized 0.22-μm-pore-size polycarbonate syringe filter (Millipore, Saint-Quentin-en-Yvelines, France).

Time-kill assay. Bottles of 20 ml of 7H9sB containing imipenem, amikacin, and avibactam alone or in combination were inoculated with exponentially growing bacteria of *M. abscessus* CIP104536 S (7×10^6 CFU/ml) and incubated with shaking (150 rpm) at 30°C for 72 h. Bacteria were enumerated at 0, 48, and 72 h by plating serial dilutions prepared in sterile saline solution on lysogeny broth (LB) plates. The plates were incubated for 4 days at 30°C. The detection limit was 2 $\log_{10}$ CFU/ml. The experiments were performed in triplicate.

Activity of imipenem alone or in combination with amikacin and avibactam in THP-1 macrophages. The activity of antibiotics was studied as previously described (15). Briefly, THP-1 cells were seeded into 24-well plates (5×10^5 cells per 1-ml well), differentiated for 24 h, and infected with *M. abscessus* CIP104536 S at a multiplicity of infection of 10 for 3 h. Imipenem (8 and 32 μg/ml), amikacin (8 μg/ml), and avibactam (16 μg/ml) alone or in combination were added to each well. The plates were incubated with 5% CO_2 at 37°C for 2 days. Macrophages were lysed with deionized water. Dilutions were plated onto LB agar plates, and the CFU were enumerated after 4 days of incubation at 30°C. The experiments were performed in triplicate.

Determination of bla_{Mab} mRNA by qRT-PCR. Extraction of bacterial RNA from *M. abscessus*-infected macrophages was performed as previously described (24). Briefly, bacterial cells were harvested after different times postinfection, washed once with Dulbecco's modified Eagle's medium alone, and resuspended in 4 M guanidine thiocyanate solution containing β-mercaptoethanol. RNA was extracted and cDNA was prepared as previously described (24). See Table S1 in the supplemental material for the sequences of the primers used for qRT-PCR. Controls without reverse transcriptase were used with each RNA sample to rule out DNA contamination (24). *sigA* gene RNA was included as an internal control.

Mouse anti-Bla_{Mab} antibodies. Purified Bla_{Mab} (12) was subcutaneously injected into five BALB/c mice (Janvier, France) (20 μg per mouse) with incomplete Freund's adjuvant (1/1, vol/vol) on days 1, 28, and 57. At 1 week after days 28 and 57, blood samples were obtained from the retroorbital plexus, centrifuged, and stored at −20°C until use. All procedures were performed according to institutional and national ethical guidelines under agreement 92-033-01 (Préfecture des Hauts-de-Seine, Boulogne-Billancourt, France).

Immunodetection of Bla_{Mab} in protein extracts. Bacterial pellets were obtained from *M. abscessus* cells grown *in vitro* and in human macrophages as previously described (24). Briefly, bacteria were resuspended in 5 ml of cooled phosphate-buffered saline (PBS) containing 1% Triton X-100 for 5 min at room temperature. Then, 2.5 ml of cold PBS was added and the suspension was centrifuged at $2,500 \times g$ for 15 min. Bacteria were resuspended in 0.5 ml Tris-buffered saline (TBS) and lysed by sonication 3 times for 30 s each time with 1-min cooling intervals on ice. After centrifugation at $14,000 \times g$ for 10 min, the protein concentration was determined using the Bradford assay with bovine serum albumin as the standard. Proteins in crude extracts (3 μg) were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), and Bla_{Mab} was detected with mouse anti-Bla_{Mab} antiserum (1/6,000). A peroxidase-conjugated goat anti-mouse immunoglobulin antibody (IgG; 1/4,000) was used as the secondary antibody. Rat anti-KasA antibodies were used as a loading control (25).

Evaluation of imipenem in a zebrafish model of *M. abscessus* infection. The zebrafish model of *M. abscessus* infection was used to assess the *in vivo* activity of imipenem alone or in combination with avibactam. The TdTomato-expressing *M. abscessus* CIP104536 R derivative was injected in zebrafish embryos according to procedures described earlier (26). Briefly, systemic infections were carried by the injection of 150 CFU into the caudal vein of embryos at 30 h postfertilization. Infected larvae were exposed to various imipenem concentrations (180 and 360 μg/ml) alone or in combination with avibactam at 50 μg/ml. Drug-containing water was renewed daily for 5 days from day 1 to day 6 postinfection. The viability of the infected embryos was evaluated daily by assessment of cardiac activity. The zebrafish experiments were conducted in accordance with the guidelines from the European Union for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and approved by the Direction Sanitaire et Vétérinaire de l'Hérault et Comité d'Ethique pour l'Expérimentation Animale de la Région Languedoc Roussillon under reference number CEEA-LR-1145.

Statistical analysis. The Mann-Whitney U test and the Kruskal-Wallis test were used to compare the intracellular activities of the antibiotics. For the zebrafish infection model, experiments were performed at least in triplicate. Data from the replicates were pooled for the construction and comparison of survival curves. The efficacy of imipenem alone or in combination with avibactam was compared using the log-rank test. All statistical analyses were performed with EPI Info software (version 7.1.3; Centers for Disease Control and Prevention, Atlanta, GA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02440-16.

TEXT S1, PDF file, 0.05 MB.

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SUPPLEMENTARY TABLES

TABLE S1 Sequence of the primers

Primer	Sequence
F-MAB2875-Q-PCR	5’- TCCATTCGGGCAGCAGTTC -3’
R-MAB2875-Q-PCR	5’- GTCGGCTTACCTTTGGTGG -3’
F-SigA	5’- TCCGAGAAAGACAAGGCTTC -3’
R-SigA	5’- CCAGCTCGACTTCCTCTTCG -3’

TABLE S2 Statistical analysis of the killing activity of imipenem (IPM) alone or in combination with avibactam (AVI) and amikacin (AMK) against *M. abscessus* CIP104536 S.

		P value for the indicated comparison (Mann-Whitney U test)										
		control	AMK32	IPM8	IPM32	AMK32+AVI4	IPM8+AMK32	IPM8+AVI4	IPM32+AMK32	IPM32+AVI4	IPM8+AMK32+AVI4	IPM32+AMK32+AVI4
		(9.44)	(6.33)	(5.09)	(4.58)	(6.58)	(4.50)	(4.92)	(4.12)	(4.48)	(3.57)	(2.48)
control	(9.44)	NA	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495
AMK32	(6.33)		NA	0.2752	0.0495	0.5127	0.0495	0.1266	0.0495	0.0495	0.0495	0.0495
IPM8	(5.09)			NA	0.1266	0.0495	0.1266	0.5127	0.1266	0.1266	0.0495	0.0495
IPM32	(4.58)				NA	0.0495	0.8273	0.2752	0.2752	0.8273	0.1840	0.0495
AMK32+AVI4	(6.58)					NA	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495
IPM8+AMK32	(4.50)						NA	0.5127	0.5127	0.8273	0.1266	0.0495
IPM8+AVI4	(4.92)							NA	0.1266	0.3758	0.0495	0.0495
IPM32+AMK32	(4.12)								NA	0.5127	0.8273	0.3758
IPM32+AVI4	(4.48)									NA	0.1266	0.0495
IPM8+AMK32+AVI4	(3.57)										NA	0.2752
IPM32+AMK32+AVI4	(2.48)											NA

TABLE S3 Statistical analysis of the intracellular activity of imipenem (IPM) alone or in combination with avibactam (AVI) and amikacin (AMK) against *M. abscessus* CIP104536 S.

		P value for the indicated comparison (Mann-Whitney U test)											
		control	AVI16	AMK8	IPM8	IPM32	AMK8+AVI16	IPM8+AMK8	IPM8+AVI16	IPM32+AMK8	IPM32+AVI16	IPM8+AMK8+AVI16	IPM32+AMK8+AVI16
		(102)	(151)	(8.00)	(4.54)	(1.57)	(104)	(4.96)	(1.13)	(1.14)	(0.10)	(0.54)	(0.07)
control	(102)	NA	0.1266	0.0495	0.0495	0.0495	0.8273	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495
AVI16	(151)		NA	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495
AMK8	(8.00)			NA	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495
IPM8	(4.54)				NA	0.0495	0.0495	0.5127	0.0495	0.0495	0.0495	0.0495	0.0495
IPM32	(1.57)					NA	0.0495	0.0495	0.1266	0.0495	0.0495	0.0495	0.0495
AMK8+AVI16	(104)						NA	0.0495	0.0495	0.0495	0.0495	0.0495	0.0495
IPM8+AMK8	(4.96)							NA	0.0495	0.0495	0.0495	0.0495	0.0495
IPM8+AVI16	(1.13)								NA	0.8273	0.0495	0.0495	0.0495
IPM32+AMK8	(1.14)									NA	0.0495	0.0495	0.0495
IPM32+AVI16	(0.10)										NA	0.0495	0.2752
IPM8+AMK32+AVI16	(0.54)											NA	0.0495
IPM32+AMK32+AVI16	(0.07)												NA