# β-Lactamase inhibition by avibactam in *Mycobacterium abscessus*

Vincent Dubée[1–3], Audrey Bernut[4,5]†, Mélanie Cortes[1–3]†, Tiffany Lesne[4,5], Delphine Dorchene[1–3],
Anne-Laure Lefebvre[1–3], Jean-Emmanuel Hugonnet[1–3], Laurent Gutmann[1–3,6], Jean-Luc Mainardi[1–3,6],
Jean-Louis Herrmann[7,8], Jean-Louis Gaillard[7,8], Laurent Kremer[4,5] and Michel Arthur[1–3]*

[1]INSERM, UMR_S 1138, Centre de Recherche des Cordeliers, F-75006, Paris, France; [2]Université Paris Descartes, Sorbonne Paris Cité, UMR_S
1138, Centre de Recherche des Cordeliers, F-75006 Paris, France; [3]Sorbonne Universités, UPMC Univ Paris 06, UMR_S 1138, Centre de
Recherche des Cordeliers, F-75006 Paris, France; [4]Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques,
Université de Montpellier 2 et 1, CNRS, UMR 5235, Montpellier, France; [5]INSERM, DIMNP, Montpellier, France; [6]Assistance Publique-Hôpitaux
de Paris, Service de Microbiologie, Hôpital Européen Georges Pompidou, Paris, France; [7]EA3647 – EPIM, Université de Versailles
St-Quentin-en-Yvelines et UFR des Sciences de la Santé, Montigny-le-Bretonneux, France; [8]Assistance Publique - Hôpitaux de Paris (AP-HP),
Service de Microbiologie, Hôpital Raymond Poincaré, Garches, France

*Corresponding author. LRMA, Equipe 12, Centre de Recherche des Cordeliers, 15 rue de l'Ecole de Médecine, 75270 Paris cedex 06, France.
Tel: +33-1-44-27-54-55; E-mail: michel.arthur@crc.jussieu.fr
†Equal contribution.

**Objectives:** Two β-lactams, cefoxitin and imipenem, are part of the reference treatment for pulmonary infections with *Mycobacterium abscessus*. *M. abscessus* has recently been shown to produce a broad-spectrum β-lactamase, $Bla_{Mab}$, indicating that the combination of β-lactams with a $Bla_{Mab}$ inhibitor may improve treatment efficacy. The objectives of this study were to evaluate the impact of $Bla_{Mab}$ production on the efficacy of β-lactams *in vitro* and to assess the benefit of $Bla_{Mab}$ inhibition on the activity of β-lactams intracellularly and in an animal model.

**Methods:** We analysed the mechanism and kinetics of $Bla_{Mab}$ inactivation by avibactam, a non-β-lactam β-lactamase inhibitor currently in Phase III of development, in combination with ceftazidime for the treatment of serious infections due to Gram-negative bacteria. We then deleted the gene encoding $Bla_{Mab}$ to assess the extent of $Bla_{Mab}$ inhibition by avibactam based on a comparison of the impact of chemical and genetic inactivation. Finally, the efficacy of amoxicillin in combination with avibactam was evaluated in cultured human macrophages and in a zebrafish model of *M. abscessus* infection.

**Results:** We showed that avibactam efficiently inactivated $Bla_{Mab}$ via the reversible formation of a covalent adduct. An inhibition of $Bla_{Mab}$ by avibactam was observed in both infected macrophages and zebrafish.

**Conclusions:** Our data identify avibactam as the first efficient inhibitor of $Bla_{Mab}$ and strongly suggest that β-lactamase inhibition should be evaluated to provide improved therapeutic options for *M. abscessus* infections.

**Keywords:** non-tuberculous mycobacteria, *M. abscessus*, therapy, β-lactams, cystic fibrosis

## Introduction

Since the individualization of *Mycobacterium abscessus* as a distinct species in 1992,[1] this fast-growing *Mycobacterium* has been recognized as an important pathogen.[2] *M. abscessus* produces numerous virulence factors[3] and causes a wide spectrum of diseases. Skin and soft tissue infections following tattooing[4] and accidental or iatrogenic inoculation[5] generally resolve with antibiotic therapy.[6] Pulmonary diseases have a poorer prognosis, especially in the context of cystic fibrosis or severe gastrointestinal reflux.[7,8] The treatment of these infections is difficult owing to

the limited number of active antibiotics that are available. *M. abscessus* is intrinsically resistant to antituberculous agents.[6] Clarithromycin cannot be uniformly recommended as a first-line drug since *erm* RNA methylase genes conferring macrolide resistance have been detected in members of the *M. abscessus* complex.[9] The gene is functional in the subspecies *abscessus*, whereas an internal deletion is present in members of the *bolletii* subspecies that had previously been classified in the *massiliense* species.[10] The treatment of *M. abscessus* infections therefore relies on a few antibiotics including amikacin, linezolid, tigecycline and the parenteral β-lactams imipenem and cefoxitin. The latter

drugs have moderate *in vitro* activity, with MICs of 4 and 32 mg/L, respectively.[11] Most other β-lactams have no appreciable *in vitro* activity.[12]

Resistance to β-lactams in mycobacteria results from the combination of several mechanisms, which have not been well characterized in *M. abscessus*. Impermeability of the mycomembrane has been shown to hamper the activity of cephalosporins in *Mycobacterium chelonae*.[13] Substitution of the classical β-lactam targets, i.e. penicillin-binding proteins, by L,D-transpeptidases[14] may further decrease the activity of penicillins and cephalosporins.[15] As shown in *Mycobacterium tuberculosis*,[16,17] the production of a chromosome-encoded Ambler class A β-lactamase (Bla_Mab) may participate in β-lactam resistance.[12]

In *M. tuberculosis*, irreversible β-lactamase inhibition by clavulanate improves the activity of β-lactams,[18] and the combination of clavulanate with meropenem is sporadically used for XDR tuberculosis.[19,20] However, *M. abscessus* Bla_Mab is not inhibited by clavulanate, sulbactam or tazobactam and, strikingly, these drugs are in fact hydrolysed by the β-lactamase.[12] We show here that avibactam, a non-β-lactam β-lactamase inhibitor currently being evaluated in Phase III trials in combination with ceftazidime for the treatment of infections due to Gram-negative bacteria (http://clinicaltrials.gov/ct2/results?term=avibactam&Search=Search), is a potent inhibitor of Bla_Mab *in vitro*. A Bla_Mab-deficient mutant was constructed to assess the extent of β-lactamase inhibition *in vitro*, in cultured macrophages[21] and in a recently developed zebrafish model of *M. abscessus* infection.[22] These approaches show that Bla_Mab is the major determinant of β-lactam resistance in *M. abscessus* and that β-lactamase inhibition should be considered to improve the treatment of *M. abscessus* infections.

## Materials and methods

### Strains, growth conditions and in vitro susceptibility testing

Derivatives of reference strain *M. abscessus* CIP104536 (ATCC 19977) with a rough (R)[23] and a smooth (S)[24] morphotype were grown in Middlebrook 7H9 broth supplemented with 10% (v/v) oleic acid, albumin, dextrose, catalase (OADC; BD-Difco) and 0.05% (v/v) Tween 80 (Sigma) (7H9sB) at 30°C with shaking (150 rpm). The R morphotype was used in the zebrafish since the S form is avirulent in this model. The S morphotype was used for the macrophage model since this form is easier to manipulate and to enumerate. To construct the Δbla_Mab mutants, the bla_Mab gene (MAB_2875) of the *M. abscessus* CIP104536 R and S morphotypes was replaced by a zeocin resistance gene using one-step homologous recombination[25] (see Figure S1 and Supplementary methods section, available as Supplementary data at *JAC* Online). *M. abscessus* CIP104536 R harbouring plasmid pTEC27, which encodes the tdTomato fluorescent protein, was cultured in 7H9sB supplemented with 500 mg/L of hygromycin.[26] Clinical isolates of *M. abscessus* belonging to the *abscessus* and *bolletii* subspecies were obtained from a previously described collection.[11] The MICs of the β-lactams were determined using the microdilution method[25] in 96-well round-bottom microplates as described in the Supplementary data.

### Biochemical analysis of Bla_Mab inactivation by avibactam

The cloning, production in *Escherichia coli* BL21 (DE3) and purification of a recombinant form of Bla_Mab have been reported elsewhere.[12] The kinetic constants for Bla_Mab inhibition were determined for a two-step reaction (Figure 1a) as previously described (further details are available as Supplementary data).[27]

### Activity of combinations of β-lactams and avibactam in THP-1 macrophages

Macrophages were infected with *M. abscessus* CIP104536 S or its Δbla_Mab derivative at a multiplicity of infection of 10:1 for 3 h (further details are available as Supplementary data).[28] Amikacin (250 mg/L) was used to eliminate extracellular bacteria prior to the addition of β-lactams and avibactam. Surviving bacteria were enumerated after 2 days of incubation by plating serial dilutions of macrophage lysates on lysogeny broth with agar.

### Efficacy of amoxicillin and avibactam in a zebrafish infection model

*M. abscessus* CIP104536 R expressing tdTomato (300 cfu) were injected into the caudal vein of 30 h post-fertilization embryos of zebrafish (*Danio rerio*, 'golden' mutant) (further details are available as Supplementary data).[22] Infected larvae were transferred into 96-well plates and exposed to various concentrations of amoxicillin (512–12800 mg/L) with or without 50 mg/L avibactam in water for 5 days. The drug-containing water was renewed daily for 5 days. The viability of the infected embryos was evaluated daily by an assessment of cardiac activity, and the dissemination of *M. abscessus* was evaluated by fluorescence microscopy. All zebrafish experiments were carried out at the University Montpellier 2, according to European Union guidelines 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 and Comité d'Ethique pour l'Expérimentation Animale de la Région Languedoc Roussillon (CEEA-LR) under the reference CEEA-LR-13007.

### Statistical analysis

For the biochemical analysis of Bla_Mab inhibition, standard errors correspond to the 95% CI of the fits performed with SigmaPlot software. The results of the MIC determinations are the medians of three independent replicates. The results of the macrophage experiments are the means ± SD of at least three independent experiments. The Mann–Whitney *U*-test and Kruskall–Wallis test were used to compare the intracellular activity of the antibiotics. For the zebrafish infection model, the experiments were performed at least in triplicate. The data for the replicates were pooled for the construction and comparison of survival curves. The efficacy of the different antibiotics was compared using the log-rank test for survival and Fisher's exact test for the frequency of abscesses. All statistical analyses were performed with Epi Info™ software version 7.1.3 (CDC, Atlanta, GA, USA).

## Results

### Deletion of the bla_Mab β-lactamase gene

To explore the role of β-lactamase Bla_Mab in β-lactam resistance, the corresponding gene (MAB_2875) was deleted from the chromosome of *M. abscessus* CIP104536 (Figure S1). Extracts and whole-cell suspensions of the WT strain rapidly hydrolysed the chromogenic cephalosporin nitrocefin (Figure S2). β-Lactamase activity was not detected in mutant Δbla_Mab, indicating that Bla_Mab is the only β-lactamase produced by *M. abscessus* CIP104536.

Since the susceptibility of *M. abscessus* to β-lactams varies according to the culture medium,[11] we determined MICs both in the reference medium for susceptibility testing, CAMHB, and in the medium routinely used in research laboratories for the optimal growth of mycobacteria, Middlebrook 7H9 broth supplemented with 10% OADC and 0.05% Tween 80 (7H9sB). In both media, *M. abscessus* CIP104536 was highly resistant to all β-lactams except cefoxitin, imipenem and meropenem, as previously
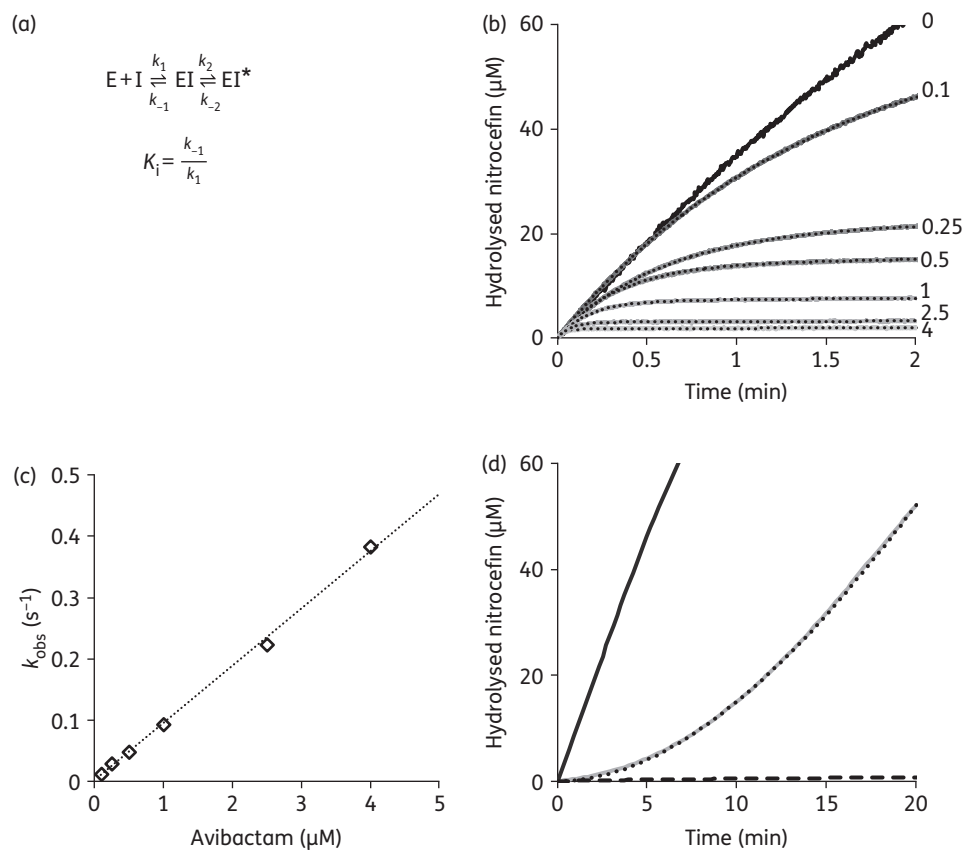
**Figure 1.** Kinetics of $Bla_{Mab}$ inhibition by avibactam. (a) Reaction scheme. E, $Bla_{Mab}$; I, avibactam; EI, non-covalent inhibitor-enzyme complex; EI*, carbamylated enzyme; $K_i$ was defined as the ratio of $k_{-1}$ over $k_1$. (b) Time-dependent inhibition of $Bla_{Mab}$ by avibactam. $Bla_{Mab}$ (0.25 nM) was incubated with nitrocefin (100 μM) and avibactam (0.1, 0.25, 0.5, 1, 2.5 and 4 μM). Progress curves (solid lines) were fitted (black dotted lines) to Equation 1 (available as Supplementary data) to obtain the pseudo-first-order rate constant $k_{obs}$. (c) Determination of carbamylation rate constant $k_2/K_i$. $k_{obs}$ was plotted as a function of avibactam concentration and $k_2/K_i$ was deduced from the slope of the resulting line according to Equation 2 (available as Supplementary data). (d) Kinetics of $Bla_{Mab}$ decarbamylation. $Bla_{Mab}$ (1 μM) was incubated with avibactam (5 μM) for 20 min. The mixture was diluted 10000-fold and recovery of enzyme activity was measured using nitrocefin (100 μM) as the substrate (grey solid curve). Under the assay conditions the concentrations of $Bla_{Mab}$ and avibactam were 100 and 500 pM, respectively. The progress curve was fitted (black dotted line) to Equation 3 (available as Supplementary data) to obtain $k_{off}$. The black solid curve corresponds to the hydrolysis of nitrocefin (100 μM) by uninhibited $Bla_{Mab}$ (100 pM). The dashed line represents spontaneous nitrocefin hydrolysis.

described (Table 1).[11,12] The deletion of $bla_{Mab}$ dramatically reduced the MICs of penicillins and first-, second- and third-generation cephalosporins (except ceftazidime), in both CAMHB and 7H9sB medium (Table 1). These results indicate that $Bla_{Mab}$ is the major determinant of high-level resistance to penicillins and most cephalosporins in *M. abscessus*. The absence of activity of ceftazidime and aztreonam against mutant $Δbla_{Mab}$ suggests that the transpeptidases of *M. abscessus* are not inhibited by these antibiotics.

### Activity of β-lactams combined with avibactam against *M. abscessus*

To explore $Bla_{Mab}$ inhibition in whole bacterial cells, we compared the MICs of β-lactams against *M. abscessus* CIP104536 in the presence or absence of avibactam (Table 1). Similar results were obtained for the S and R morphotypes. In 7H9sB medium, this β-lactamase inhibitor decreased the MICs of amoxicillin,

cefalotin, cefuroxime, cefamandole and ceftriaxone (Table 1). The addition of avibactam to the culture medium and the deletion of $bla_{Mab}$ had strikingly similar impacts on the MICs, indicating that $Bla_{Mab}$ was completely inhibited in the 7H9sB medium. Avibactam had no intrinsic antibacterial activity since the drug did not modify the MICs of β-lactams against mutant $Δbla_{Mab}$ and did not inhibit the growth of the *M. abscessus* strains (MICs >256 mg/L).

The MICs of cefoxitin and imipenem are generally higher in the reference medium, CAMHB, than in 7H9sB.[11] A comparison of MICs of additional β-lactams against the $Δbla_{Mab}$ mutant in the two media (Table 1) indicated that all β-lactams were intrinsically less active in CAMHB (2- to 8-fold). A complete inactivation of $Bla_{Mab}$ by avibactam was not achieved in CAMHB medium since the addition of the inhibitor to the growth medium did not reduce the MICs of β-lactams to the same extent as the deletion of $bla_{Mab}$. Thus, both β-lactams and avibactam were less active in CAMHB than in 7H9sB medium.

**Table 1.** MICs (mg/L) of β-lactams in CAMHB and 7H9sB media with or without 4 mg/L avibactam

| | CAMHB medium | | | | 7H9sB medium | | | |
| | CIP104536 | | $\Delta bla_{Mab}$ | | CIP104536 | | $\Delta bla_{Mab}$ | |
| β-Lactam | −avibactam | +avibactam | −avibactam | +avibactam | −avibactam | +avibactam | −avibactam | +avibactam |
|---|---|---|---|---|---|---|---|---|
| Amoxicillin | >256 | 256 | 8 | 16 | >256 | 8 | 4 | 4 |
| Cefalotin | >256 | 256 | 16 | 16 | >256 | 8 | 4 | 8 |
| Cefuroxime | 256 | 32 | 16 | 32 | 32 | 8 | 4 | 8 |
| Cefamandole | >256 | 64 | 16 | 16 | 128 | 8 | 4 | 4 |
| Ceftriaxone | >256 | 256 | 64 | 32 | 64 | 8 | 8 | 8 |
| Ceftazidime | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Cefoxitin | 32 | 32 | 32 | 32 | 16 | 8 | 8 | 16 |
| Imipenem | 8 | 8 | 4 | 4 | 4 | 2 | 2 | 2 |
| Meropenem | 16 | 8 | 8 | 8 | 4 | 4 | 4 | 4 |
| Aztreonam | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |

Results are the medians of three independent experiments performed with the S morphotype of *M. abscessus* CIP104536 and its $\Delta bla_{Mab}$ derivative. MICs observed for the R variant of *M. abscessus* CIP104536 were not different (data not shown).
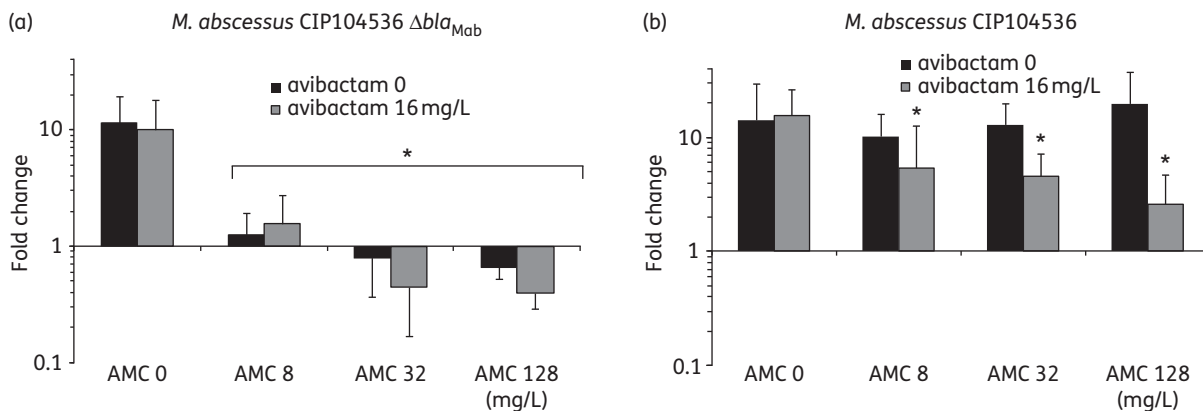


**Figure 2.** Intracellular activity of amoxicillin (AMC) combined with avibactam. Fold change in cfu between 0 and 2 days post-infection. Results are the means ± SEM of at least three independent experiments. *$P < 0.05$ versus untreated control using the Mann–Whitney *U*-test.

### In vitro inhibition of Bla$_{Mab}$

Avibactam is unique among β-lactamase inhibitors since carbamylation of the enzyme active-site serine is fully reversible according to the reaction scheme depicted in Figure 1(a). The efficacy of this mode of inhibition depends upon both the carbamylation and decarbamylation rates. Since the inhibition of *M. abscessus* β-lactamase has not been previously investigated, we have purified a soluble form of Bla$_{Mab}$ and determined its inhibition kinetics using nitrocefin as the substrate (Figure 1). Avibactam inhibited Bla$_{Mab}$ in a time-dependent manner with a carbamylation rate constant ($k_2/K_i$) of $4.9 \pm 1.2 \times 10^5$ M$^{-1}$ s$^{-1}$ (Figure 1b and c), close to that observed for the prototypic Ambler class A β-lactamase TEM-1 ($1.6 \pm 1.2 \times 10^5$ M$^{-1}$ s$^{-1}$).[27] Decarbamylation occurred with a rate constant of $0.047 \pm 0.0001$ min$^{-1}$ (Figure 1d), implying that half the enzyme recovered its activity within 15 min in the absence of avibactam. This value is also similar to the decarbamylation rate constant reported for TEM-1 ($0.045 \pm 0.022$ min$^{-1}$).[27] The hydrolysis of avibactam has been reported for a single β-lactamase, KPC-2, from *Klebsiella pneumoniae*.[29] In our study, the hydrolysis of avibactam by Bla$_{Mab}$ was not detected by mass spectrometry (data not shown). Together, these results indicate that avibactam inhibits β-lactamases from *M. abscessus* and from the Enterobacteriaceae with similar efficiencies.

### Intramacrophagic activity of amoxicillin combined with avibactam

Macrophages were infected with *M. abscessus* CIP104536 S and its $\Delta bla_{Mab}$ derivative and exposed to various concentrations of amoxicillin and avibactam; the surviving bacteria were enumerated after 2 days of incubation by plating serial dilutions of macrophage lysates. In the absence of antibiotic, *M. abscessus* CIP104536 S and its $\Delta bla_{Mab}$ derivative grew in THP-1-derived macrophages, leading to a 10-fold increase in the cfu numbers in 2 days (Figure 2).
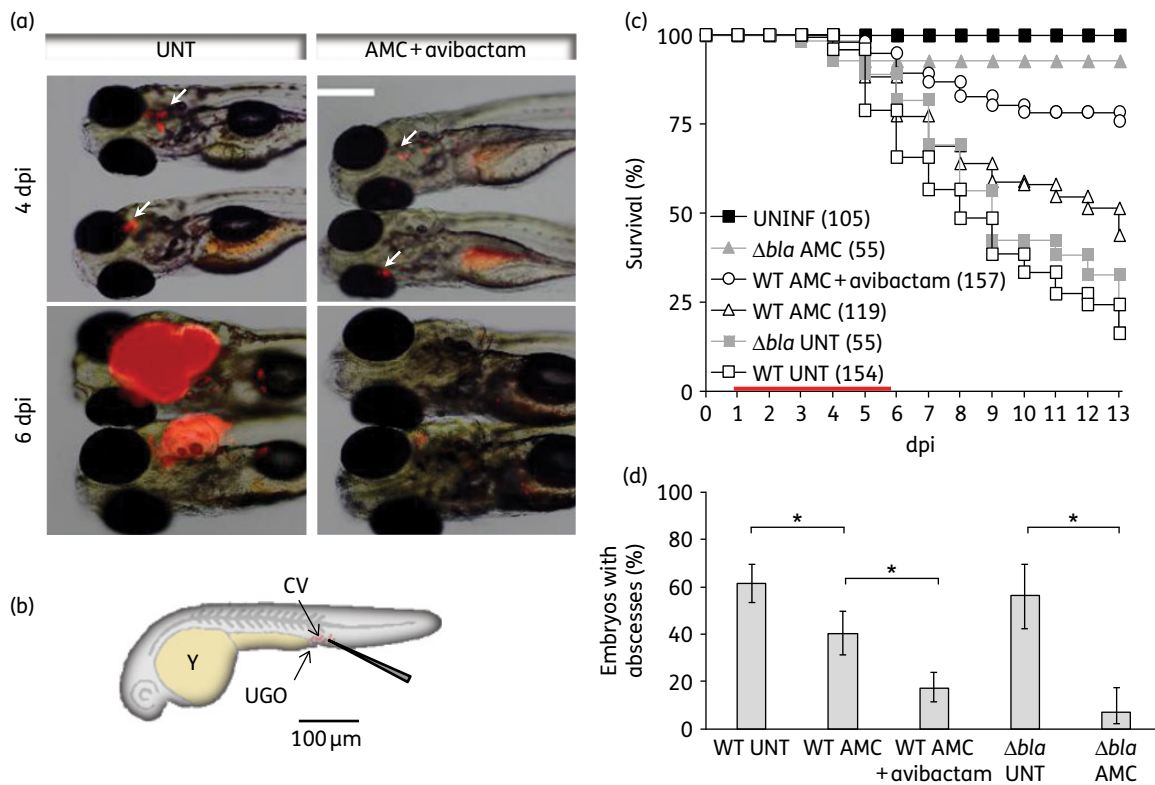
**Figure 3.** Efficacy of the amoxicillin/avibactam combination in *M. abscessus*-infected zebrafish. (a) Representative fluorescence and transmission overlay of embryos infected by *M. abscessus* CIP10536 R expressing tdTomato red fluorescent protein. Embryos were infected at 30 h post-fertilization (hpf) and treated from Day 1 to Day 6 post-infection with amoxicillin (AMC; 5000 mg/L) and avibactam (50 mg/L) or left untreated (UNT). The panels show representative overlays obtained 4 and 6 days post-infection (dpi). The yolk is autofluorescent. Arrows indicate the presence of red fluorescent abscesses. Scale bar = 300 μm. (b) Cartoon of a 30 hpf embryo showing the injection site (grey arrowhead) in the caudal vein (CV) just behind the urogenital opening (UGO). Y, yolk. (c) Survival of embryos infected by *M. abscessus*. Animals were infected with WT *M. abscessus* CIP10536 R (WT) and its $\Delta bla_{Mab}$ derivative ($\Delta bla$) or left uninfected (UNINF). Infected animals were treated with 5 mg/mL AMC or the drug combination (AMC+avibactam) or left UNT. The red bar represents the period of exposure to the drugs. The number of embryos in each group is indicated in parentheses. (d) Proportions of infected embryos with abscesses. Error bars are 95% CIs. *$P < 0.001$. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Amoxicillin at 8, 32 and 128 mg/L prevented the intracellular growth of the $\Delta bla_{Mab}$ mutant (Figure 2a). Amoxicillin alone had no effect against the WT CIP104536 strain, whereas this drug in combination with avibactam (16 mg/L) exhibited dose-dependent activity (Figure 2b). However, comparison with the $\Delta bla_{Mab}$ mutant indicated that a complete inactivation of $Bla_{Mab}$ by avibactam was not achieved inside the macrophages.

### Efficacy of avibactam in a zebrafish model of *M. abscessus* infection

A recently developed zebrafish model[22] of *M. abscessus* infection was used to assess the *in vivo* inhibition of $Bla_{Mab}$ by avibactam. In this model, zebrafish embryos infected by *M. abscessus* CIP104536 R developed abscesses mainly located in the CNS within 4 days after infection (Figure 3a and b), leading to a rapid decrease in survival that began at day 5 post-infection (Figure 3c). Amoxicillin at the highest non-toxic concentration (Figure S3) significantly decreased the mortality of infected larvae (Figure 3c, $P < 10^{-4}$). The addition of avibactam (50 mg/L) further increased the survival ($P < 10^{-4}$). The efficacy of amoxicillin and of the amoxicillin/avibactam combination was also evaluated by

enumerating infected larvae with abscesses by live fluorescence microscopy (Figure 3d). Amoxicillin alone was active in reducing the proportion of embryos with abscesses (62% of embryos infected by the WT strain and left untreated developed abscesses versus 40% for embryos infected by the WT strain and treated with amoxicillin, $P = 0.0006$). Avibactam improved the efficacy of amoxicillin (17%, $P = 0.00003$). Overall, these results indicate that avibactam inhibited $Bla_{Mab}$ in the conditions that prevail in the abscesses since the drug improved the efficacy of amoxicillin. As expected, amoxicillin was active in the absence of avibactam against mutant $\Delta bla_{Mab}$ (Figure 3c and d).

### Activity of the amoxicillin/avibactam combination against a collection of *M. abscessus* isolates

The variability in the *in vitro* activity of β-lactams and avibactam was evaluated in a collection of 16 *M. abscessus* clinical isolates from a previous study (Table S1).[11] Amoxicillin alone did not inhibit the growth of any of the 16 isolates up to a concentration of 256 mg/L. Avibactam at 4 mg/L improved the activity of amoxicillin. The MICs of amoxicillin in the presence of avibactam were in the same range as the MICs of cefoxitin (4 to 32 mg/L versus

8 to 32 mg/L, respectively). These results indicate that amoxicillin displayed antibacterial activity against all isolates following β-lactamase inhibition by avibactam.

## Discussion

β-lactamases Bla$_{Mab}$ from *M. abscessus* and BlaC from *M. tuberculosis* have similar broad hydrolysis spectra.[12,17] In contrast, the β-lactamases have opposite behaviours with respect to their interaction with β-lactamase inhibitors since Bla$_{Mab}$ efficiently hydrolyses clavulanate, sulbactam and tazobactam,[12] whereas BlaC is inhibited by these drugs, including irreversible inactivation in the case of clavulanate.[30] In this study, we show that avibactam is a potent inhibitor of purified Bla$_{Mab}$ (Figure 1), whereas the inhibition of BlaC by this non-β-lactam inhibitor was detected only at high concentrations.[31] The identification of avibactam as an efficient inhibitor of Bla$_{Mab}$ prompted us to evaluate the benefit of β-lactamase inhibition on the activity of β-lactams against *M. abscessus in vitro*, intracellularly and in an animal model.

In order to evaluate the efficacy of avibactam for the *in vitro* inhibition of Bla$_{Mab}$, we constructed a β-lactamase-deficient mutant of *M. abscessus*. In 7H9sB medium, the MICs of β-lactams against this mutant were very close to those observed for the parental strain in the presence of 4 mg/L avibactam, indicating that a low concentration of inhibitor is sufficient to fully inhibit Bla$_{Mab}$ (Table 1). An inhibition of Bla$_{Mab}$ was also observed in a set of 16 clinical isolates (Table S1). In the absence of functional Bla$_{Mab}$, several representatives of the three main classes of β-lactams (penams, cephalosporins and carbapenems) were found to be active against *M. abscessus*. This observation indicates that the cross-linking step of peptidoglycan synthesis, which involves two pathways in *M. abscessus*,[32] is inhibited by a large spectrum of β-lactams, although most of them are devoid of antibacterial activity due to their hydrolysis by Bla$_{Mab}$. In 7H9sB medium, the MICs of the two drugs used for the treatment of *M. abscessus* infections, imipenem and cefoxitin, were 4 and 16 mg/L. In the absence of functional Bla$_{Mab}$, the MICs of several additional β-lactams belonged to the same range (4–8 mg/L). These results indicate that avibactam may potentially extend the therapeutic options among β-lactams for the treatment of *M. abscessus* infections. Of note, the MICs of all the β-lactams were relatively high even in the absence of Bla$_{Mab}$. Thus, low permeability may also contribute to intrinsic β-lactam resistance in *M. abscessus*, as previously proposed for *M. chelonae*.[13]

In order to determine the intracellular activity of avibactam, we assessed the efficacy of the amoxicillin/avibactam combination in macrophages (Figure 2). Because amoxicillin is one of the best substrates of Bla$_{Mab}$,[12] this β-lactam provides a stringent assay for the intracellular inhibition of Bla$_{Mab}$ by avibactam. Amoxicillin inhibited the intracellular growth of mutant Δbla$_{Mab}$ at concentrations close to the MIC in 7H9sB medium, indicating that the drug penetrates into the macrophages and is intracellularly active (Figure 2a). An assay of the amoxicillin/avibactam combination against the parental strain revealed the intracellular inhibition of Bla$_{Mab}$ by avibactam (Figure 2b). However, the inhibition of the β-lactamase was partial since the number of intracellular bacteria was higher for chemical (Figure 2b) than for genetic (Figure 2a) enzyme inactivation. This may reflect a limited penetration of avibactam into the macrophages. Of note, avibactam has been reported to be active in THP-1 monocytes infected by *Pseudomonas aeruginosa*.[33]

The *in vivo* evaluation of β-lactam efficacy in rodent models of *M. abscessus* infection suffers from several limitations, including the spontaneous clearance of infection and difficulties in achieving sufficient blood levels, especially with carbapenems, which are rapidly hydrolysed by renal dehydropeptidase.[34–36] A zebrafish model of *M. abscessus* infection has recently been developed[24] and used for the *in vivo* evaluation of drug efficacy.[34] We used this model, which offers several advantages, including speed, low cost and ethical acceptability, to assess the inhibition of Bla$_{Mab}$ in infectious forms of *M. abscessus*. Avibactam efficiently increased survival and decreased abscess formation in zebrafish larvae treated with amoxicillin (Figure 3). Thus, the inhibition of Bla$_{Mab}$ increased the *in vivo* activity of amoxicillin.

### Conclusions

*M. abscessus* lung infections have a poor prognosis, especially in the context of underlying pulmonary diseases such as cystic fibrosis. Active β-lactams currently include only cefoxitin and carbapenems, which are parenteral drugs. Here we show that the spectrum of active β-lactams can be extended by the inhibition of *M. abscessus* β-lactamase Bla$_{Mab}$. This could potentially provide alternatives to imipenem and cefoxitin with better PK/PD parameters. Since the approved inhibitors, clavulanate, sulbactam and tazobactam, are hydrolysed by Bla$_{Mab}$, avibactam is the only inhibitor that can be currently considered. We have shown that this drug efficiently inhibits Bla$_{Mab}$ by the reversible formation of a covalent adduct and is active intracellularly and in the zebrafish model. In humans, avibactam has shown an excellent tolerance profile in Phase I and II studies.[37–39] The drug penetrates into epithelial lining fluid[40] and its activity is not impaired by pulmonary surfactant.[41] The combination of avibactam and ceftazidime is currently in Phase III trials for treatment of nosocomial pulmonary infections and complicated urinary tract and intra-abdominal infections. Unfortunately, this combination is not relevant to the treatment of *M. abscessus* infections due to the lack of activity of ceftazidime. In addition, avibactam is being developed as a parenteral drug. The development of an orally bioavailable combination of a β-lactam with a Bla$_{Mab}$ inhibitor is an attractive approach to improve therapeutic options for *M. abscessus* infections. The excellent *in vitro* activity of avibactam and the unique mode of action of this inhibitor suggest that derivatives of this drug should be considered for the development of this type of combination.

## Transparency declarations

J.-L. M. has received consultant fees from AstraZeneca (scientific advisor for ceftaroline). In addition, AstraZeneca covered expenses for J.-L. M. to attend the Fifty-fourth Interscience Conference on Antimicrobial Agents and Chemotherapy. All other authors: none to declare.

## Supplementary data

Supplementary methods, Figures S1 to S3 and Table S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

## References

**1** Kusunoki S, Ezaki T. Proposal of *Mycobacterium peregrinum* sp. nov., nom. rev., and elevation of *Mycobacterium chelonae* subsp. *abscessus* (Kubica *et al*.) to species status: *Mycobacterium abscessus* comb. nov. *Int J Syst Bacteriol* 1992; **42**: 240–5.

**2** Medjahed H, Gaillard JL, Reyrat JM. *Mycobacterium abscessus*: a new player in the mycobacterial field. *Trends Microbiol* 2010; **18**: 117–23.

**3** Ripoll F, Pasek S, Schenowitz C *et al*. Non mycobacterial virulence genes in the genome of the emerging pathogen *Mycobacterium abscessus*. *PLoS One* 2009; **4**: e5660.

**4** Falsey RR, Kinzer MH, Hurst S *et al*. Cutaneous inoculation of nontuberculous mycobacteria during professional tattooing: a case series and epidemiologic study. *Clin Infect Dis* 2013; **57**: e143–7.

**5** Ryu HJ, Kim WJ, Oh CH *et al*. Iatrogenic *Mycobacterium abscessus* infection associated with acupuncture: clinical manifestations and its treatment. *Int J Dermatol* 2005; **44**: 846–50.

**6** Griffith DE, Aksamit T, Brown-Elliott BA *et al*. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med* 2007; **175**: 367–416.

**7** Griffith DE, Girard WM, Wallace RJ Jr. Clinical features of pulmonary disease caused by rapidly growing mycobacteria. An analysis of 154 patients. *Am Rev Respir Dis* 1993; **147**: 1271–8.

**8** Koh WJ, Lee JH, Kwon YS *et al*. Prevalence of gastroesophageal reflux disease in patients with nontuberculous mycobacterial lung disease. *Chest* 2007; **131**: 1825–30.

**9** Nash KA, Brown-Elliott BA, Wallace RJ Jr. A novel gene, *erm*(41), confers inducible macrolide resistance to clinical isolates of *Mycobacterium abscessus* but is absent from *Mycobacterium chelonae*. *Antimicrob Agents Chemother* 2009; **53**: 1367–76.

**10** Choi GE, Shin SJ, Won CJ *et al*. Macrolide treatment for *Mycobacterium abscessus* and *Mycobacterium massiliense* infection and inducible resistance. *Am J Respir Crit Care Med* 2012; **186**: 917–25.

**11** Lavollay M, Dubée V, Heym B *et al*. *In vitro* activity of cefoxitin and imipenem against *Mycobacterium abscessus* complex. *Clin Microbiol Infect* 2014; **20**: O297–300.

**12** Soroka D, Dubée V, Soulier-Escrihuela O *et al*. Characterization of broad-spectrum *Mycobacterium abscessus* class A β-lactamase. *J Antimicrob Chemother* 2014; **69**: 691–6.

**13** Jarlier V, Gutmann L, Nikaido H. Interplay of cell wall barrier and β-lactamase activity determines high resistance to β-lactam antibiotics in *Mycobacterium chelonae*. *Antimicrob Agents Chemother* 1991; **35**: 1937–9.

**14** Lavollay M, Arthur M, Fourgeaud M *et al*. The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. *J Bacteriol* 2008; **190**: 4360–6.

**15** Triboulet S, Dubee V, Lecoq L *et al*. Kinetic features of L,D-transpeptidase inactivation critical for β-lactam antibacterial activity. *PLoS One* 2013; **8**: e67831.

**16** Flores AR, Parsons LM, Pavelka MS Jr. Genetic analysis of the β-lactamases of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* and susceptibility to β-lactam antibiotics. *Microbiology* 2005; **151**: 521–32.

**17** Wang F, Cassidy C, Sacchettini JC. Crystal structure and activity studies of the *Mycobacterium tuberculosis* β-lactamase reveal its critical role in resistance to β-lactam antibiotics. *Antimicrob Agents Chemother* 2006; **50**: 2762–71.

**18** Hugonnet JE, Tremblay LW, Boshoff HI *et al*. Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. *Science* 2009; **323**: 1215–8.

**19** Dauby N, Muylle I, Mouchet F *et al*. Meropenem/clavulanate and linezolid treatment for extensively drug-resistant tuberculosis. *Pediatr Infect Dis J* 2011; **30**: 812–3.

**20** Payen MC, De Wit S, Martin C *et al*. Clinical use of the meropenem-clavulanate combination for extensively drug-resistant tuberculosis. *Int J Tuberc Lung Dis* 2012; **16**: 558–60.

**21** Roux AL, Ray A, Pawlik A *et al*. Overexpression of proinflammatory TLR-2-signalling lipoproteins in hypervirulent mycobacterial variants. *Cell Microbiol* 2011; **13**: 692–704.

**22** Bernut A, Herrmann JL, Kissa K *et al*. *Mycobacterium abscessus* cording prevents phagocytosis and promotes abscess formation. *Proc Natl Acad Sci USA* 2014; **111**: E943–52.

**23** Catherinot E, Clarissou J, Etienne G *et al*. Hypervirulence of a rough variant of the *Mycobacterium abscessus* type strain. *Infect Immun* 2007; **75**: 1055–8.

**24** Moore M, Frerichs JB. An unusual acid-fast infection of the knee with subcutaneous, abscess-like lesions of the gluteal region; report of a case with a study of the organism, *Mycobacterium abscessus*, n. sp. *J Invest Dermatol* 1953; **20**: 133–69.

**25** Medjahed H, Reyrat JM. Construction of *Mycobacterium abscessus* defined glycopeptidolipid mutants: comparison of genetic tools. *Appl Environ Microbiol* 2009; **75**: 1331–8.

**26** Clinical and Laboratory Standards Institute. *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes—Second Edition: Approved Standard M24-A2*. CLSI, Wayne, PA, USA, 2011.

**27** Ehmann DE, Jahic H, Ross PL *et al*. Avibactam is a covalent, reversible, non-β-lactam β-lactamase inhibitor. *Proc Natl Acad Sci USA* 2012; **109**: 11663–8.

**28** Sarkar S, Sarkar D. Potential use of nitrate reductase as a biomarker for the identification of active and dormant inhibitors of *Mycobacterium tuberculosis* in a THP1 infection model. *J Biomol Screen* 2012; **17**: 966–73.

**29** Ehmann DE, Jahic H, Ross PL *et al*. Kinetics of avibactam inhibition against class A, C, and D β-lactamases. *J Biol Chem* 2013; **288**: 27960–71.

**30** Hugonnet JE, Blanchard JS. Irreversible inhibition of the *Mycobacterium tuberculosis* β-lactamase by clavulanate. *Biochemistry* 2007; **46**: 11998–2004.

**31** Xu H, Hazra S, Blanchard JS. NXL104 irreversibly inhibits the β-lactamase from *Mycobacterium tuberculosis*. *Biochemistry* 2012; **51**: 4551–7.

**32** Lavollay M, Fourgeaud M, Herrmann JL *et al*. The peptidoglycan of *Mycobacterium abscessus* is predominantly cross-linked by L,D-transpeptidases. *J Bacteriol* 2011; **193**: 778–82.

**33** Buyck J, Luyck C, Van Bambeke F *et al*. Activity and phramacodynamic (PD) evaluation of ceftazidime-avibactam (CAZ-AVI) against extracellular and intracellular forms of CAZ-susceptible and CAZ-resistant *Pseudomonas aeruginosa* (PA). In: *Abstracts of the Fifty-first Interscience Conference on*

*Antimicrobial Agents and Chemotherapy, Denver, CO, 2013*. Abstract A-1021. American Society for Microbiology, Washington, DC, USA.

**34** Bernut A, Le Moigne V, Lesne T *et al. In vivo* assessment of drug efficacy against *Mycobacterium abscessus* using the embryonic zebrafish test system. *Antimicrob Agents Chemother* 2014; **58**: 4054–63.

**35** Fukasawa M, Sumita Y, Harabe ET *et al.* Stability of meropenem and effect of 1 β-methyl substitution on its stability in the presence of renal dehydropeptidase I. *Antimicrob Agents Chemother* 1992; **36**: 1577–9.

**36** England K, Boshoff HI, Arora K *et al.* Meropenem-clavulanic acid shows activity against *Mycobacterium tuberculosis in vivo. Antimicrob Agents Chemother* 2012; **56**: 3384–7.

**37** Riccobene TA, Su SF, Rank D. Single- and multiple-dose study to determine the safety, tolerability, and pharmacokinetics of ceftaroline fosamil in combination with avibactam in healthy subjects. *Antimicrob Agents Chemother* 2013; **57**: 1496–504.

**38** Vazquez JA, Gonzalez Patzan LD, Stricklin D *et al.* Efficacy and safety of ceftazidime-avibactam versus imipenem-cilastatin in the treatment of complicated urinary tract infections, including acute pyelonephritis, in hospitalized adults: results of a prospective, investigator-blinded, randomized study. *Curr Med Res Opin* 2012; **28**: 1921–31.

**39** Lucasti C, Popescu I, Ramesh MK *et al.* Comparative study of the efficacy and safety of ceftazidime/avibactam plus metronidazole versus meropenem in the treatment of complicated intra-abdominal infections in hospitalized adults: results of a randomized, double-blind, Phase II trial. *J Antimicrob Chemother* 2013; **68**: 1183–92.

**40** Nicolau DP, Siew L, Armstrong J *et al.* Concentration of avibactam (AVI) and ceftazidime (CAZ) in plasma and epithelial lining fluid (ELF) in healthy volunteers. In: Ab*stracts of the Fifty-third Interscience Conference on Antimicrobial Agents and Chemotherapy, Denver, CO, USA, 2013*. Abstract A-1027. American Society for Microbiology, Washington, DC, USA.

**41** Dallow J, Otterson LG, Huband MD *et al.* Microbiological interaction studies between ceftazidime-avibactam and lung surfactant and between ceftazidime-avibactam and antibacterial agents of other classes. *Int J Antimicrob Agents* 2014; **44**: 552–6.