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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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Running Head: Activity of imipenem against *Mycobacterium abscessus*

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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 despite production of the broad spectrum β-lactamase Bla_{Mab}. We determine whether inhibition of Bla_{Mab} by avibactam improves the activity of imipenem against *M. abscessus.* 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 qRT-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) achieving a 3.2 or 4.3 Log_{10}-reduction in the number of cfus at 72 h, respectively. The triple combination achieved significant intracellular killing with a bacterial survival rate of 54% and 7% for the low (8 µg/ml) and high (32 µg/ml) dosage 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 improves 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. *In vitro* evaluation of imipenem may underestimate the impact of Bla_{Mab} since production of the β-lactamase is inducible in macrophages.**

45 In the context of cystic fibrosis, *Mycobacterium abscessus* has emerged in recent years as an
46 important opportunistic lung pathogen increasingly responsible for mortality (1-5). These
47 infections are extremely difficult to treat and to eradicate as *M. abscessus* is naturally
48 resistant to most antibiotics, including antituberculous agents (6, 7). The recommended
49 treatment for pulmonary infections relies on combination of a macrolide (clarithromycin or
50 azithromycin), an aminoglycoside (amikacin) and an intravenous β-lactam (cefoxitin or
51 imipenem) for at least 12 to 16 months (6, 8). Resistance to macrolide, present in 40 to 60%
52 of the isolates (9), leads to a cure rate of only 25-40% (88 to 95% for macrolide-susceptible
53 isolates). Cefoxitin and imipenem, are the most active β-lactams in spite of moderate *in vitro*
54 activity with minimum inhibitory concentrations ($MICs_{50\%}$) of 32 and 16 µg/ml, respectively
55 (10).

56 We have previously shown that production of a broad-spectrum β-lactamase, Bla_{Mab}, is
57 a major determinant of β-lactam resistance in *M. abscessus* (11, 12). In contrast to the β-
58 lactamase BlaC from *M. tuberculosis*, Bla_{Mab} is not inactivated by clavulanate, sulbactam and
59 tazobactam since these inhibitors are hydrolyzed by the β-lactamase. However, Bla_{Mab} is
60 efficiently inhibited by avibactam (12), a β-lactamase inhibitor approved by the FDA in 2014
61 (13, 14). Deletion of the gene encoding Bla_{Mab} or chemical inhibition of the β-lactamase
62 activity by avibactam extend the spectrum of β-lactams active against *M. abscessus*, as
63 previously shown for an extensive evaluation of the amoxicillin-avibactam combination (12).

64 Recently, we have reported that the killing of *M. abscessus* by the combination of
65 imipenem and amikacin is significantly higher against a Bla_{Mab}-deficient mutant than against
66 the parental strain (15). In macrophages, the difference was even more pronounced since a
67 100-fold reduction in intracellular bacteria was observed for the Bla_{Mab}-deficient mutant
68 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 have evaluated whether Bla_{Mab} inhibition by avibactam could potentiate the effects of imipenem *in vitro*, in macrophages, and in zebrafish embryos. The latter 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*.

MATERIAL AND METHODS

Bacterial strains and growth conditions. *M. abscessus* CIP104536 (ATCC19977) with a smooth (S) or rough (R) morphotype and their 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, catalase (OADC; BD-Difco) and 0.05% (vol/vol) Tween 80 (Sigma-Aldrich) (7H9sB) at 30°C with shaking (150 rpm) (17).

Antibiotics. Amikacin was provided by Bristol-Myers Squibb (Rueil-Malmaison, France) and imipenem by Mylan (Saint-Priest, France). Avibactam was provided by AstraZeneca. Water was the solvent for preparing stock solutions, which were freshly prepared for each experiment and filtered using sterilized 0.22 µm polycarbonate syringe filter (Millipore, Saint-Quentin-en-Yvelines, France).

92 **Time-kill assay.** Bottles of 20 ml of 7H9sB containing imipenem, amikacin, and
93 avibactam alone or in combination were inoculated with exponentially-growing bacteria of
94 *M. abscessus* CIP104536 S (7 x 10^6 cfu/ml) and incubated with shaking (150 rpm) at 30°C for
95 72 h. Bacteria were enumerated at 0, 48, and 72 h by plating serial dilutions prepared in
96 sterile saline solution on lysogeny broth (LB) plates. Plates were incubated for 4 days at
97 30 °C. The detection limit was 2 Log_{10} cfu/ml. Experiments were performed in triplicate.

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

107 **Determination of bla_{Mab} mRNA by qRT-PCR.** Extraction of bacterial RNA from *M.*
108 *abscessus*-infected macrophages was performed as previously described (18). Briefly,
109 bacterial cells were harvested after different times post-infection, washed once with
110 Dulbecco's modified Eagle's medium alone, and resuspended in GTG 4M containing β-
111 mercaptoethanol. RNA was extracted and cDNA prepared as previously described (18). See
112 Table S1 in the supplemental material for the sequence of the primers used for qRT-PCR.
113 Controls without reverse transcriptase were done on each RNA sample to rule out DNA
114 contamination (18). The s*igA* gene RNA was included as an internal control.

Mouse anti-Bla_{Mab} antibodies. Purified Bla_{Mab} (12) was subcutaneously injected in five BALB/c mice (Janvier, France) (20 µg per mouse) with incomplete Freund's adjuvant (1/1, Vol/Vol) at D1, D28, and D57. One week after D28 and D57, blood samples were obtained from the retro-orbital plexus, centrifuged, and stored at −20°C until use. All procedures were performed according to the 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* grown *in vitro* and in human macrophages as previously described (18). Briefly, bacteria were resuspended in 5 ml of cooled PBS containing 1% triton for 5 min at room temperature. Then, 2.5 ml of cold PBS was added and the suspension was centrifuged at 2,500 x *g* for 15 min. Bacteria were resuspended in 0.5 ml Tris-Buffered Saline (TBS) and lyzed by sonication 3 × 30 s with 1 min cooling intervals on ice. After centrifugation at 14,000 x *g* for 10 min, 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, transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), and Bla_{Mab} was detected with the mouse anti-Bla_{Mab} antiserum (1/6,000). A peroxidase-conjugated goat anti-mouse antibody (IgG; 1/4,000) was used as the second antibody. Rat anti-KasA antibodies were used as a loading control (19).

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. TdTomato-expressing *M. abscessus* CIP104536 R derivative was injected in zebrafish embryos according to procedures described earlier (20). Briefly, systemic infections were carried by the injection of 150 cfus into the caudal vein of 30 h post-fertilization embryos. Infected larvae were exposed to various imipenem

concentrations (180 and 360 µg/ml) alone or in combination with avibactam 50 µg/ml. Drug-containing water was renewed daily for 5 days from day 1 to day 6 post infection. The viability of infected embryos was evaluated daily by assessment of cardiac activity. Zebrafish experiments were conducted in accordance with the guidelines from the European Union for 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 the reference CEEA-LR-1145.

Statistical analysis. The Mann-Whitney U test and the Kruskall-Wallis test were used to compare the intracellular activity of antibiotics. For the zebrafish infection model, experiments were performed at least in triplicate. Data from the replicates were pooled for construction and comparison of survival curves. 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).

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 S (15). Amikacin was tested at 4 fold the MIC (32 µg/ml). Avibactam

163 was tested at 4 µg/ml, as used for susceptibility testing in *Enterobacteriaceae* (21, 22).
164 Reductions in the Log_{10} of cfus of *M. abscessus* CIP104536 S were observed for imipenem at
165 8 µg/ml, tested alone (1.7 Log_{10}) or in combination with amikacin (2.3 Log_{10}) or avibactam
166 (1.9 Log_{10}) (Fig. 1A) (See Table S2 in the supplemental material for statistical analysis). The
167 triple combination of imipenem-amikacin-avibactam was bactericidal (less than 0.1%
168 survival), achieving a 3.2 Log_{10}-reduction in the number of cfus. The triple combination was
169 more active than imipenem plus avibactam (3.2 *versus* 1.9 Log_{10}-reduction; $P < 0.05$) but the
170 difference with imipenem plus amikacin was not significant (3.2 *versus* 2.3 Log_{10}-reduction; P
171 = 0.12). Increasing the imipenem concentration from 8 µg/ml (Fig. 1A) to 32 µg/ml (Fig. 1B)
172 moderately increased the activity of imipenem alone (1.7 *versus* 2.2 Log_{10}-reduction), of
173 imipenem combined with amikacin (2.3 *versus* 2.7 Log_{10}-reduction), and of imipenem
174 combined with avibactam (1.9 *versus* 2.3 Log_{10}-reduction) but none of these differences
175 were statistically significant. Imipenem at 32 µg/ml combined with amikacin and avibactam
176 was the most active drug combination achieving a 4.3 Log_{10}-reduction.

177 **Intramacrophage activity of imipenem alone or in combination with amikacin and**
178 **avibactam.** THP-1-derived macrophages were infected with *M. abscessus* CIP104536 S,
179 exposed to various drugs for 2 days, and surviving bacteria were enumerated by plating
180 serial dilutions of macrophage lysates (Fig. 2) (See Table S3 in the supplemental material for
181 statistical analysis). In the absence of antibiotic, *M. abscessus* CIP104536 S grew in
182 macrophages, leading to a 100-fold increase in the number of cfus at 2 days. Imipenem at 8
183 µg/ml (Fig. 2) partially prevented intramacrophage growth of *M. abscessus* CIP104536 S (4.5-
184 *versus* 100-fold increase in cfus; $P < 0.05$). Amikacin was also active (8-fold increase in cfus; P
185 < 0.05). The combination of imipenem and amikacin was not more active than imipenem
186 alone (4.5- *versus* 5.0-fold increases in cfus, respectively; $P = 0.51$). In contrast, avibactam

187 improved the activity of imipenem preventing intracellular proliferation of *M. abscessus*
188 CIP104536 S (1.1- *versus* 4.5-fold increases in cfus; $P < 0.05$). The combination of imipenem
189 (8 µg/ml), amikacin, and avibactam was the only combination that reduced the number of
190 intracellular bacteria (fold change of 0.54). This value was significantly different from that
191 obtained with imipenem combined with avibactam (0.54 *versus* 1.1; $P < 0.05$). Increasing the
192 concentration of imipenem from 8 µg/ml to 32 µg/ml improved the activity of the drug
193 tested alone (4.5- *versus* 1.6-fold change in cfus), and in combination with amikacin (5.0-
194 *versus* 1.1-fold change), avibactam (1.1- *versus* 0.1-fold change), and amikacin and
195 avibactam (0.54- *versus* 0.07-fold change). The latter differences were significant ($P < 0.05$).
196 In conclusion, avibactam significantly improved the activity of imipenem when tested both
197 with and without amikacin. Significant intracellular killing was obtained with the double and
198 triple combinations involving imipenem at 32 µg/ml and avibactam with or without
199 amikacin.

200 **Bla_{Mab} is produced at a high level within macrophages.** The expression of *bla_{Mab}* was
201 investigated at both the transcriptional and translational levels in *M. abscessus* grown *in*
202 *vitro* or proliferating in infected macrophages. Quantitative RT-PCR analyses indicated that
203 growth of *M. abscessus* within macrophages led to a twenty-fold increase in the relative
204 abundance of the *bla_{Mab}* transcript in comparison to planktonic cultures performed *in vitro* in
205 7H9B medium (Fig. 3A). Western blot analysis confirmed that Bla_{Mab} is produced at a higher
206 level in macrophages (Fig. 3B).

207 **Avibactam increases the efficacy of imipenem in the zebrafish model of *M. abscessus***
208 **infection.** The Zebrafish model of *M. abscessus* infection (16) was used to assess the *in vivo*
209 efficacy of imipenem following inhibition of Bla_{Mab} by avibactam (Fig. 4). Although imipenem
210 alone was active, avibactam further increased larval survival ($P < 0.05$ for both tested

concentrations of imipenem). These results indicate that 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.

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. 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 1B). 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* proliferating in planktonic cultures than in infected macrophages (Fig. 3). Thus, the high-level of production of Bla_{Mab} in the infected macrophages, which was confirmed by Western blot analysis, may account for

235 the fact that avibactam has a greater impact on the efficacy of imipenem intracellularly than
236 *in vitro*. The induction of *bla*$_{Mab}$ in macrophages indicates that the *in vitro* evaluation of
237 imipenem may underestimate the impact of the β-lactamase Bla$_{Mab}$. Of note, there is only a
238 two-fold difference in the MIC of imipenem against the Bla$_{Mab}$-deficient mutant of *M.*
239 *abscessus* and the parental strain (23).

240 Two β-lactams, cefoxitin and imipenem, are recommended for the treatment of
241 pulmonary infections due to *M. abscessus* (8). Currently, there is no recommendation for the
242 preferential use of one these drugs. We have previously shown that Bla$_{Mab}$ hydrolyzes
243 imipenem with a moderate but significant catalytic efficacy (3 x 10^4 M^{-1} s^{-1}) (11). Bla$_{Mab}$ is
244 4,000-fold less active for the hydrolysis of cefoxitin. The low efficacy of hydrolysis of
245 cefoxitin by Bla$_{Mab}$ is likely to be irrelevant since the killing curve assay did not reveal any
246 difference in the activity of cefoxitin, alone or in combination with amikacin, against a
247 Bla$_{Mab}$-deficient mutant of *M. abscessus* and the parental strain (15). In this study, we have
248 shown that imipenem is highly active in macrophages, but this requires combination with
249 avibactam due to the induction of *bla*$_{Mab}$. Together, these data indicate that imipenem is
250 intrinsically more active than cefoxitin although this difference is compensated by the higher
251 hydrolysis of imipenem by the β-lactamase Bla$_{Mab}$.

252 The assessments of the efficacy of drug combinations *in vitro* (Fig. 1), in macrophages
253 (Fig. 2), and in the zebrafish (Fig. 4) indicate that the triple combination of avibactam,
254 imipenem, and amikacin should be clinically evaluated, particularly in infections due to
255 clarithromycin-resistant *M. abscessus*, which are often not cured by the recommended
256 treatments (7, 24-26). Unfortunately, avibactam is currently manufactured in combination
257 with ceftazidime, which has no activity against *M. abscessus* (12). Formulation of avibactam

258 independently from any β-lactam partner would be necessary to provide cystic fibrosis

259 patients with an access to the avibactam-imipenem-amikacin combination.

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Legends to Figures

FIG 1 Time-kill curves 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 IPM at 8 µg/ml alone or in combination with AVI (4 µg/ml) and AMK (32 µg/ml). The number of cfus was determined after 0, 48 and 72 h of exposure to antibiotics. (B) Time-kill curves of IPM at 32 µg/ml alone or in combination with AVI (4 µg/ml) and AMK (32 µg/ml).

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

385 change in cfus was determined between days 0 and 2 post-infection. Bars represent
386 standard deviations.
387
388 **FIG 3** Production of Bla_{Mab} *in vitro* and in macrophages. (A) Quantification of bla_{Mab} by qRT-
389 PCR. RNA was isolated from wild-type strain CIP CIP104536 (S) grown *in vitro* in 7H9
390 medium for 48 h or in human J774 macrophages for 24 h, 48 h, 72 h, and 96 h. The *sigA*
391 rRNA was used as an internal standard. The values are the ratio of intramacrophage to *in*
392 *vitro* growth. Results are expressed as means +/- standard deviations from three
393 experiments performed in triplicate. Bars represent standard deviations. (B)
394 Immunodetection of Bla_{Mab}. Protein extracts were prepared from wild-type strain CIP
395 CIP104536 (S) and its Δbla_{Mab} derivative grown *in vitro* in 7H9 medium for 48 h or in human
396 J774 macrophages for 1 h, 24 h, and 48 h. Proteins (3 µg) were separated by 15% SDS-PAGE.
397 Immunodetection was performed with a mouse immune serum specific for Bla_{Mab} and a
398 peroxidase-conjugated goat anti-mouse antibody. Immuno-detection of KasA was used as a
399 loading control.
400
401 **FIG 4** Efficacy of imipenem alone or in combination with avibactam in zebrafish embryos
402 infected by *M. abscessus* CIP104536 R expressing TdTomato. Embryos (60/300 per group)
403 were infected at 30 h post fertilization and exposed to imipenem (IMI) from day 1 to day 6
404 post infection (dpi) at two concentrations (180 and 360 µg/ml, panels A and B, respectively)
405 alone or in combination with avibactam at 50 µg/ml (AVI). Control animals were left
406 untreated (UNT). The survival of animals was monitored each day post infection and the
407 results were expressed as the % of the larval survival. *$P < 0.05$.