



Bedaquiline Inhibits the ATP Synthase in *Mycobacterium abscessus* and Is Effective in Infected Zebrafish

Christian Dupont,^a Albertus Viljoen,^a Sangeeta Thomas,^c
Françoise Roquet-Banères,^a Jean-Louis Herrmann,^d Kevin Pethe,^c
Laurent Kremer^{a,b}

Centre National de la Recherche Scientifique UMR9004, Institut de Recherche en Infectiologie de Montpellier (IRIM), Université de Montpellier, Montpellier, France^a; INSERM, IRIM, Montpellier, France^b; Lee Kong Chian School of Medicine and School of Biological Sciences, Nanyang Technological University Singapore, Singapore^c; INSERM, UMR1173, Université de Versailles Saint-Quentin-en-Yvelines, Montigny-le-Bretonneux, France^d

ABSTRACT Pulmonary infections caused by *Mycobacterium abscessus* are emerging as a global threat, especially in cystic fibrosis patients. Further intensifying the concern of *M. abscessus* infection is the recent evidence of human-to-human transmission of the infection. *M. abscessus* is a naturally multidrug-resistant fast-growing pathogen for which pharmacological options are limited. Repurposing antitubercular drugs represents an attractive option for the development of chemotherapeutic alternatives against *M. abscessus* infections. Bedaquiline (BDQ), an ATP synthase inhibitor, has recently been approved for the treatment of multidrug-resistant tuberculosis. Herein, we show that BDQ has a very low MIC against a vast panel of clinical isolates. Despite being bacteriostatic *in vitro*, BDQ was highly efficacious in a zebrafish model of *M. abscessus* infection. Remarkably, a very short period of treatment was sufficient to protect the infected larvae from *M. abscessus*-induced killing. This was corroborated with reduced numbers of abscesses and cords, considered to be major pathophysiological signs in infected zebrafish. Mode-of-action studies revealed that BDQ triggered a rapid depletion of ATP in *M. abscessus in vitro*, consistent with the drug targeting the F_oF₁ ATP synthase. Importantly, despite a failure to select *in vitro* for spontaneous mutants that are highly resistant to BDQ, the transfer of single nucleotide polymorphisms leading to D29V or A64P substitutions in *atpE* conferred high resistance, thus resolving the target of BDQ in *M. abscessus*. Overall, this study indicates that BDQ is active against *M. abscessus in vitro* and *in vivo* and should be considered for clinical use against the difficult-to-manage *M. abscessus* pulmonary infections.

KEYWORDS *Mycobacterium abscessus*, bedaquiline, zebrafish, therapeutic activity, resistance, ATP synthase, AtpE, drug resistance mechanisms

M*ycobacterium abscessus* pulmonary disease is a significant cause of morbidity and mortality among patients with preexisting lung conditions, such as bronchiectasis, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF). Whole-genome sequencing of *M. abscessus* in CF patients indicated human-to-human transmission of this infection (1, 2). Together with members of the *Mycobacterium avium* complex (MAC), *M. abscessus* represents the most frequent nontuberculous mycobacterium (NTM) respiratory pathogen. Unfortunately, there are no predictable or reliably effective treatment strategies for pulmonary infections caused by *M. abscessus* (3). MAC and *M. abscessus* lung diseases, similarly to multidrug-resistant tuberculosis (MDR-TB), are very difficult to treat due to limited therapeutic options, particularly when current

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Address correspondence to Laurent Kremer,
laurent.kremer@irim.cnrs.fr.

C.D. and A.V. contributed equally to this article.

therapy fails. The American Thoracic Society recommends a treatment regimen consisting of a combination of a macrolide (clarithromycin or azithromycin), an aminoglycoside (amikacin), and a β -lactam (cefoxitin or imipenem) for a period of 1 year (4). *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* possess an *erm*(41) RNA methylase gene that confers inducible resistance to macrolides (5). Intravenous antibiotics are an essential element for *M. abscessus* lung disease treatment. Unfortunately, oral medication options are limited against *M. abscessus* diseases. Therefore, new chemotherapeutic options, particularly those given orally, are urgently needed to improve *M. abscessus* treatment outcomes, particularly for clarithromycin-resistant *M. abscessus* lung diseases.

To alleviate the initiation of new *de novo* chemical screens against *M. abscessus*, “cross-screen” approaches based on existing data from previous TB screens have recently been applied to identify new pharmacological entities that are active against *M. abscessus*. This confirmed that TB drug discovery platforms could easily be exploited to screen for *M. abscessus*-active compounds. This led, for instance, to the identification of a piperidinol-based compound (6) or thiacetazone (TAC) derivatives (7), which exhibit potent activity against *M. abscessus*. However, the quest for discovering potent novel active drugs against *M. abscessus* could also rely on repurposing TB drugs.

Bedaquiline (BDQ; code names TMC207 and R207910), which was approved by the Food and Drug Administration and the European Medicines Agency for the treatment of MDR-TB (8), is a diarylquinoline antibiotic that acts through inhibition of the essential F_0F_1 ATP synthase. There is now clear evidence that BDQ targets the c subunit of the ATP synthase (9–11). Biochemical and X-ray crystallographic studies indicated that BDQ is likely to prevent the rotor ring from acting as an ion shuttle (12). Recent biochemical and genetic studies also proposed that BDQ inhibits mycobacterial F-ATP synthase via another mechanism involving the ϵ subunit of the enzyme in addition to binding to its c subunit (13, 14). Interestingly, BDQ has also been shown to be more effective than currently existing antimycobacterial agents in treating *Mycobacterium ulcerans* in a mouse model of infection and has shown promise as a salvage therapy for *M. avium* and *M. abscessus* (15, 16). Very few experimental models have been developed for rapidly growing mycobacterial infections, which hampers *in vivo* drug susceptibility testing. Hence, a limited number of animal models for evaluating antibiotic activity against *M. abscessus* infection have been reported recently, mostly based on the use of immunocompromised mice (17–21). Two independent studies that evaluated the efficacy of BDQ to reduce the *M. abscessus* loads in those models led to conflictual conclusions. Studies conducted in gamma interferon knockout (GKO) mice have shown a potent benefit of BDQ treatment in reducing bacterial loads (19), whereas work in nude mice failed to show a decrease in bacillary loads in the lungs and prevention of death, in contrast to cefoxitin (20). We previously reported the usefulness of zebrafish as a preclinical model to evaluate in real time the efficacy of antibiotics, particularly clarithromycin and imipenem, against *M. abscessus* in living infected vertebrates (22–24). This biological system can be complementary to murine models, as it permits *in vivo* imaging, at the spatiotemporal level, of the effects of drug treatment on the infection process.

Thus, to determine whether BDQ has a place in the management of *M. abscessus* infection, we investigated the potential antimicrobial efficacy of BDQ against the *M. abscessus* complex *in vitro* against *M. abscessus* clinical isolates, particularly from CF patients, as well as in zebrafish embryos. This study was also undertaken to determine the mode of action and the mechanism(s) of resistance of BDQ in *M. abscessus*. We provide evidence that, as observed in *M. tuberculosis*, BDQ triggers rapid ATP depletion in *M. abscessus*, and high levels of resistance are associated with mutations in *atpE*.

RESULTS

BDQ inhibits *M. abscessus* growth *in vitro*. The exposure of exponentially growing *M. abscessus* to increasing concentrations of BDQ, corresponding to 2 \times , 8 \times , and 64 \times the MIC (0.125 μ g/ml), showed important growth inhibition (Fig. 1). However, since the

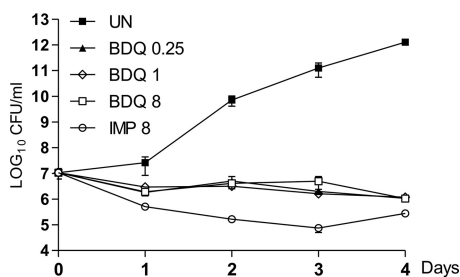


FIG 1 *In vitro* activity of bedaquiline. *M. abscessus* CIP104536^T (S) was exposed either to 8 μ g of imipenem (IMP) or to increasing concentrations of BDQ (from 2 to 64 \times the MIC) in CaMH broth at 30°C. At various time points, bacteria were plated on LB agar and further incubated at 30°C for 4 days prior to CFU determination. Results are expressed as the mean \pm standard error of the mean (SEM) of triplicates and are representative of two independent experiments. UN, untreated cultures.

CFU numbers over the 4-day period of treatment were only slightly below those of the inoculum and remained constant over time, these results suggest that BDQ exerts a bacteriostatic effect *in vitro* against *M. abscessus*. Exposure to imipenem, an active β -lactam drug against *M. abscessus* (25), was associated with a more pronounced killing effect (Fig. 1).

Activity of BDQ against *M. abscessus* isolates. The activity of BDQ was next tested using a large set of clinical isolates. The *M. abscessus* group is classified into three groups: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*, and this distinction is of clinical relevance, as these subspecies respond differently to antibiotics (26, 27). BDQ exhibited potent activity against the different strains isolated from either CF patients or non-CF patients, with MICs ranging from 0.031 to 0.125 μ g/ml (Table 1). The same strains also exhibited various susceptibility profiles to imipenem, one of the most widely used drugs in clinical settings, ranging from 8 to 32 μ g/ml. The smooth and rough morphotypes of *M. abscessus*, here referred to as S and R, respectively, were also equally sensitive to BDQ. Overall, these results demonstrate that BDQ exerts very strong activity against the *M. abscessus* complex, including isolates from CF patients.

***In vivo* susceptibility of *M. abscessus* to bedaquiline.** Animal models are currently limited to study host immunity and pathogenesis unless very large doses of bacilli are given intravenously. If small doses are given, there is little evidence that a productive infection is even fully established. Consequently, better models are required to elucidate pathogenesis and to enable new drugs for *M. abscessus* infections to be tested. This led to the recent development of the zebrafish model to assess the suitability and sensitivity of clinically relevant drugs in *M. abscessus*-infected embryos (6, 21–24). Small bacterial doses can be used in this model to allow visualizing, in a dose- and time-dependent manner, the dynamics of infection and physiopathological markers, such as cords and abscesses, in the presence of an active compound (22). The injection of a small inoculum allows administration of homogenous bacterial suspensions without obstructing the needle during the microinjection procedure. Therefore, we adapted a previously designed protocol (22) to assess the activity of BDQ against *M. abscessus* in zebrafish larvae. Red fluorescent tdTomato-expressing *M. abscessus* (R variant) was injected in the caudal vein of embryos at 30 h postfertilization (hpf) and transferred to 24-well plates. BDQ was then directly added at 1 day postinfection (dpi) to the water containing the infected zebrafish, and the BDQ-supplemented water was then changed on a daily basis for 3 days. In preliminary experiments, noninfected embryos were exposed to increasing concentrations of BDQ and observed under a microscope. No signs of toxicity-induced killing or developmental abnormalities were recorded in the presence of 3 μ g/ml BDQ (data not shown). When infected embryos were exposed for 3 days to the lowest (1 μ g/ml) concentration of BDQ tested, a significant increased survival rate was observed compared to the untreated group of embryos and was as efficient as treatment with 360 μ g/ml imipenem (Fig. 2A). Exposure to a higher dose of

TABLE 1 Comparison of activities of BDQ and imipenem against clinical isolates from CF and non-CF patients

Strain	Morphotype	Source	MIC ($\mu\text{g/ml}$) ^a	
			BDQ	IMP
<i>M. abscessus</i> subsp. <i>abscessus</i>				
CIP104536 ^T	S	Non-CF	0.062	16
3321	S	Non-CF	0.062	32
1298	S	CF	0.031	16
2587	S	CF	0.062	16
2069	S	Non-CF	0.062	8
CF	S	CF	0.062	16
2524	R	CF	0.125	32
2648	R	CF	0.062	32
3022	R	Non-CF	0.062	16
5175	R	CF	0.062	32
CIP104536 ^T	R	Non-CF	0.062	16
<i>M. abscessus</i> subsp. <i>massiliense</i>				
CIP108297 ^T	R	Addison disease	0.062	32
210	R	CF	0.125	32
179	R	CF	0.062	8
CIP108297 ^T	S	Addison disease	0.062	32
140	S	CF	0.062	32
185	S	CF	0.125	16
107	S	CF	0.125	32
122	S	CF	0.125	16
120	S	CF	0.062	16
212	S	CF	0.125	32
100	S	CF	0.062	16
111	S	CF	0.062	16
<i>M. abscessus</i> subsp. <i>bolletii</i>				
CIP108541 ^T	S	Nonreported	0.062	8
114	S	CF	0.125	16
17	S	CF	0.031	32
116	S	CF	0.125	16
97	S	CF	0.125	16
112	R	CF	0.062	16
19	R	Non-CF	0.125	32
10	R	Nonreported	0.062	32
108	R	CF	0.125	32

^aThe MIC was determined in cation-adjusted Mueller-Hinton broth for different subspecies belonging to the *M. abscessus* complex. BDQ, bedaquiline; IMP, imipenem.

BDQ (3 $\mu\text{g/ml}$) further extended the life span of infected zebrafish and protected around 80% of the infected embryos at 13 dpi (Fig. 2A). This indicates that BDQ is very efficient in this zebrafish test system against *M. abscessus* infection. We next investigated whether reducing the duration of treatment would affect the protective efficacy of BDQ. Infected embryos were treated with 3 $\mu\text{g/ml}$ BDQ for 1, 2, or 3 days, and the efficacy of the treatment was determined by monitoring the killing curves of the various treatments. Figure 2B clearly indicates that 24 or 48 h of treatment resulted in high survival rates against *M. abscessus*, although the percentage of survival was lower than for embryos treated for 3 days. Overall, these results suggest that short treatment with BDQ is sufficient to confer high protection levels against *M. abscessus* infection.

Previous studies have shown that virulence of the rough strain of *M. abscessus* in zebrafish is correlated with the presence of abscesses, particularly in the central nervous system, and extracellular cords, which due to their size prevent the bacilli from being phagocytosed by macrophages (28, 29). To investigate whether the high survival rates are associated with decreased pathophysiological symptoms upon drug treatment, the percentages of abscesses and cords were determined. In agreement with previous studies, exposure to imipenem was associated with a significant reduction in the numbers of abscesses (Fig. 2C) and cords (Fig. 2D) (22). Importantly, and similarly to imipenem, exposure of infected embryos to 3 $\mu\text{g/ml}$ BDQ for either 24, 48, or 72 h

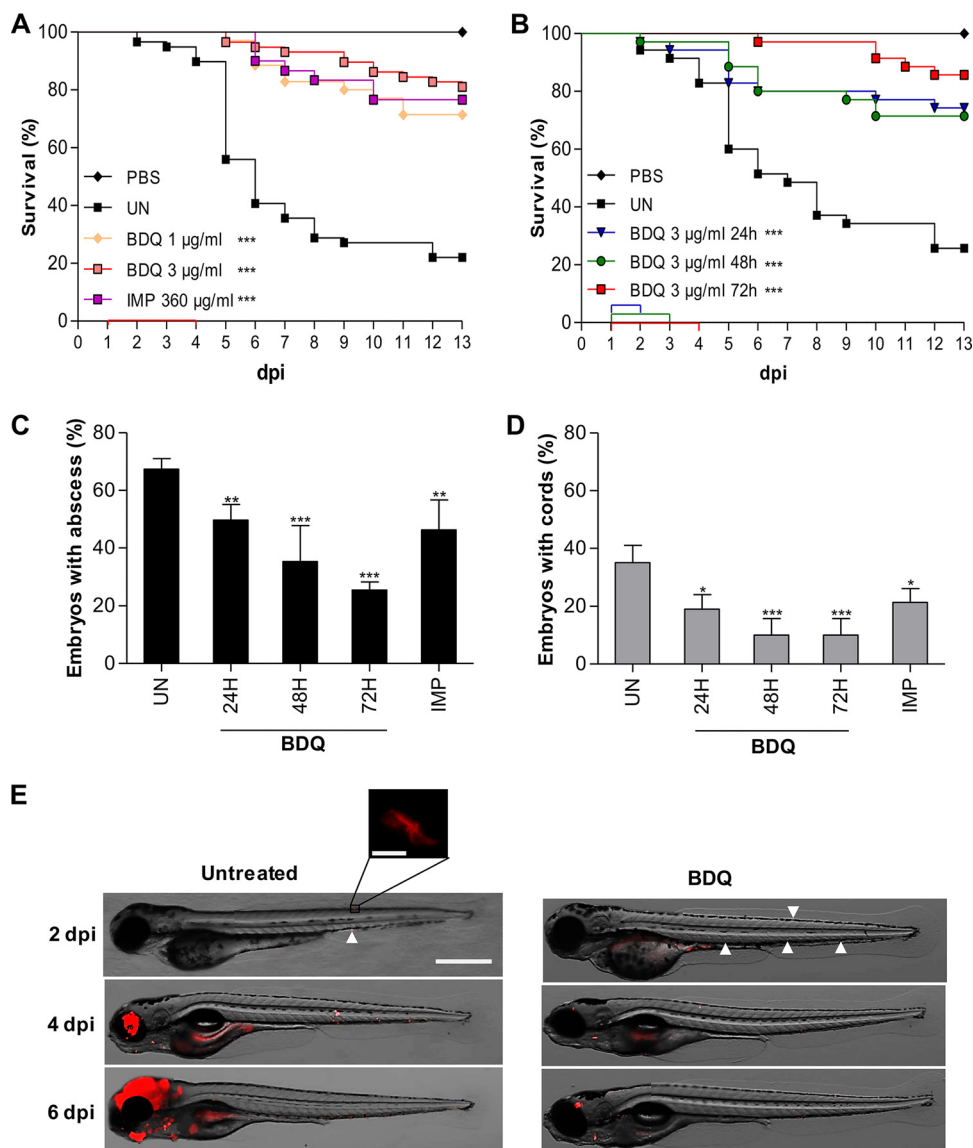


FIG 2 Activity of BDQ against *M. abscessus* in infected zebrafish embryos. Survival curve of embryos infected with 50 to 270 CFU of rough *M. abscessus* CIP104536T expressing tdTomato without treatment (UN) or treated with 1 or 3 µg/ml BDQ or with 360 µg/ml imipenem (IMP) for 3 days (A) and with 3 µg/ml BDQ for 1, 2, or 3 days (B). Embryos injected with PBS were used as controls for injections. Treatment started at 1 dpi. The duration of the treatment is indicated with a colored line along the x axis. Curves are representative of two independent experiments. Data were subjected to the log rank statistical test ($n = 20$ to 30); ***, $P < 0.001$. (C) Frequency of abscesses in whole untreated or drug-treated embryos was recorded at 5 dpi (50 to 270 CFU, $n = 30$). Data are representative of three experiments, and statistical analysis was done using Fisher's exact test; **, $P < 0.01$; ***, $P < 0.001$. (D) Frequency of cords in whole untreated or drug-treated embryos was recorded at 5 dpi (50 to 270 CFU, $n = 30$). Data are representative of three experiments, and statistical analysis was done using Fisher's exact test; *, $P < 0.05$; ***, $P < 0.001$. (E) Spatiotemporal visualization of the infection by *M. abscessus* expressing tdTomato in untreated or BDQ-treated embryos at 2, 4, and 6 dpi. The representative fluorescence and transmission overlays of whole embryos are shown (scale bar, 200 µm), with the inset displaying a cord at higher resolution (scale bar, 10 µm). White arrowheads at 2 dpi indicate individual bacilli or small bacterial clumps.

was accompanied by a significant decrease in the frequency of abscesses (Fig. 2C) and cords (Fig. 2D). This decrease in the physiopathological signs of BDQ-treated larvae was corroborated by whole-embryo imaging (Fig. 2E).

Together, these results suggest that BDQ exerts a therapeutic effect by preventing the development of abscesses/cords and protecting the embryos from bacterial killing.

Bedaquiline inhibits ATP production in *M. abscessus*. BDQ has been identified as a potent and specific inhibitor of mycobacterial ATP synthase, thereby validating

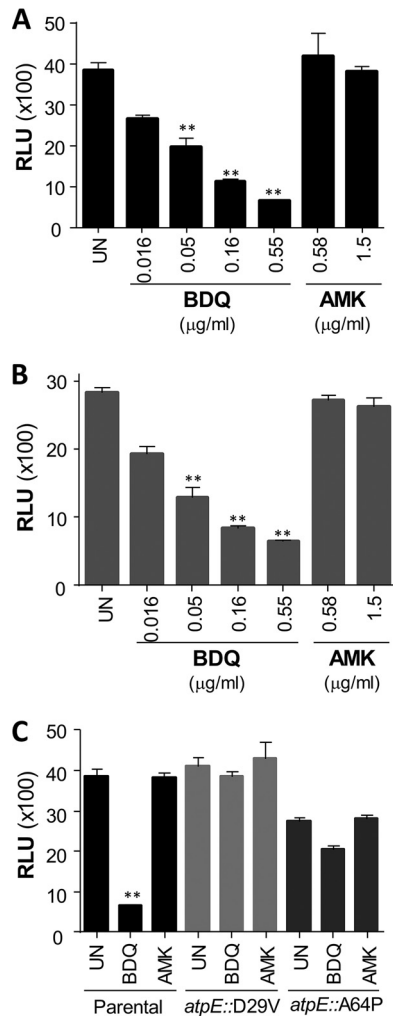


FIG 3 Inhibition of ATP synthesis by BDQ in *M. abscessus*. ATP levels were measured in the *M. abscessus* rough (A) and smooth (B) variants incubated with a dose range of either BDQ or amikacin (AMK) for 180 min. (C) ATP levels were determined in the parental CIP104536^T smooth strain and the CIP104536^T*atpE::D29V* and CIP104536^T*atpE::A64P* derivatives treated with 0.55 μg/ml BDQ or 1.5 μg/ml AMK. Statistical analysis was performed using one-way analysis of variance (ANOVA), with Dunnett's multiple-comparison test. **, $P < 0.01$.

oxidative phosphorylation as a target pathway for antimycobacterial drug development (9–11). Thus, we investigated whether BDQ inhibits ATP homeostasis in *M. abscessus* and found that BDQ induced a rapid dose-dependent depletion of the intracellular ATP pool as soon as 180 min posttreatment (Fig. 3). Under similar experimental conditions, amikacin did not have any effect on ATP synthesis, indicating that rapid ATP depletion is specific to BDQ. Comparable results were obtained in both the rough and smooth variants of *M. abscessus*. Thus, it can be inferred that the F_0F_1 ATP synthase is the primary target of BDQ in *M. abscessus*.

Genetic validation of AtpE as the target of BDQ in *M. abscessus*. The c subunit of the ATP synthase of *M. abscessus* (MAB_1448) shares a very high sequence identity with its *M. tuberculosis* (92%) and *M. smegmatis* (85%) homologues, and particularly, a full conservation of the sequence in the transmembrane region where BDQ has been proposed to bind (9, 12). In addition, Ala63 and Asp32, the major residues previously shown to participate in BDQ resistance in *M. tuberculosis* and *M. smegmatis* (9, 10), respectively, are conserved in *M. abscessus* (Fig. 4A). To validate *atpE* as a specific target of BDQ, several *atpE* alleles were cloned under the control of the constitutive *hsp60* promoter in the replicative pMV261 to allow overexpression of the wild-type (pAtpE) or

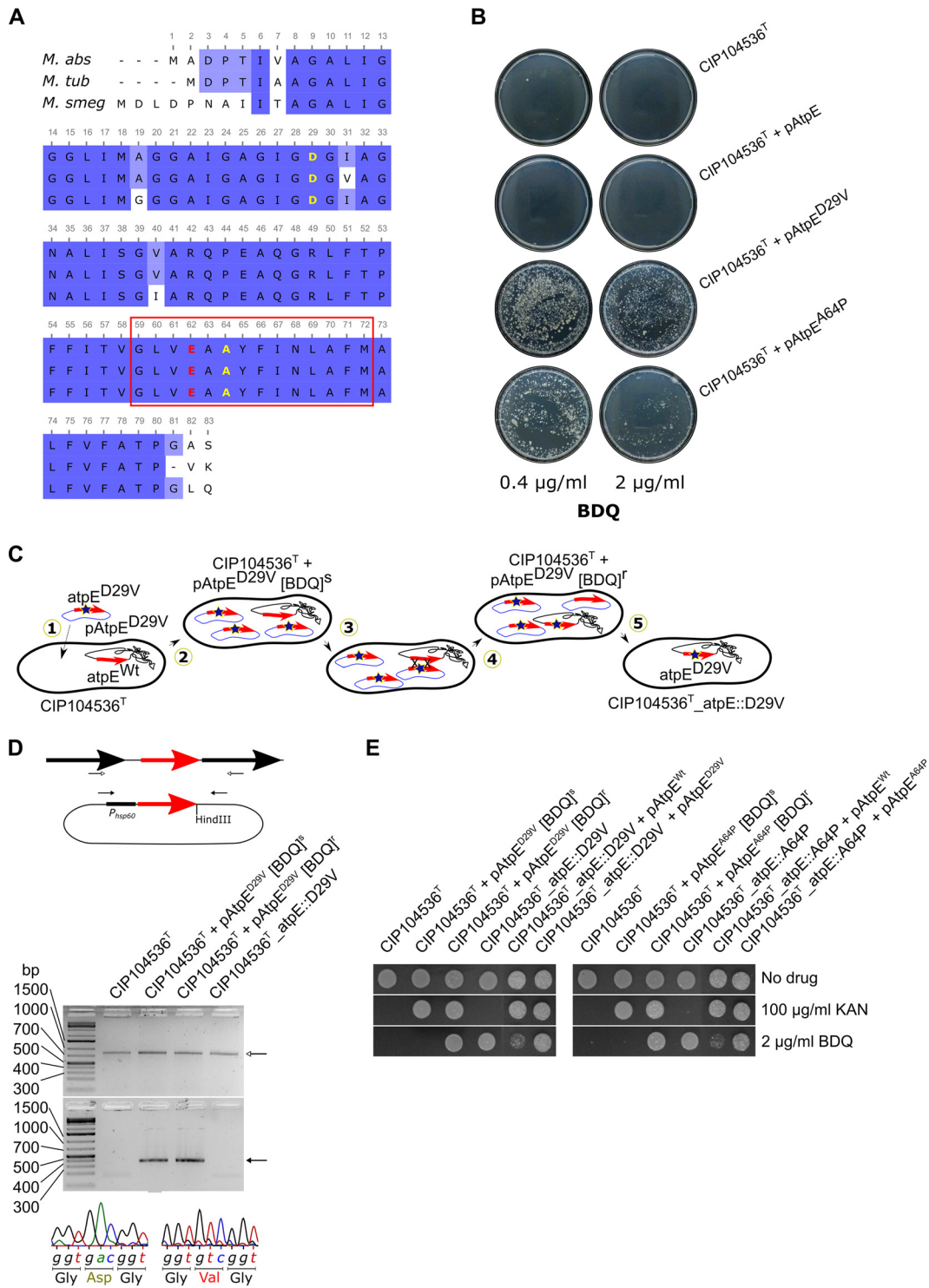


FIG 4 Generation of *M. abscessus* strains that are highly resistant to BDQ through homologous recombination in the *atpE* locus. (A) Multiple alignment showing high amino acid identity between homologues of the ATP synthase c subunit in *M. abscessus* (*M. abs*), *M. tuberculosis* (*M. tub*), and *M. smegmatis* (*M. smeg*). The red rectangle comprises the peptide sequence of the c subunit, previously shown to interact with BDQ via numerous Van der Waals interactions. The proton-binding glutamic acid is indicated in red. Nonsynonymous mutations of the conserved D29 and A64 residues were previously identified in *M. tuberculosis* and *M. smegmatis* and shown to confer resistance in these strains probably by creating steric clashes with BDQ, preventing it from binding. (B) Selection of acquired resistance to BDQ in *M. abscessus* carrying the pMV261 derivatives with wild-type (pAtpE) or mutated *atpE* alleles (pAtpE^{D29V} and pAtpE^{A64P}). Ten out of 10 such colonies arising from either plasmid were confirmed to have undergone homologous recombination between plasmid-borne and chromosomal *atpE* loci. (C) Introduction of SNPs into the chromosomal *atpE* locus conferring BDQ resistance is accomplished through the following steps: in step 1, *M. abscessus* is first transformed with a multicopy vector harboring a mutated *atpE* allele (red arrow). In step 2, (Continued on next page)

TABLE 2 MICs of BDQ against *M. abscessus* S strains carrying single point mutations in *atpE* either on a pMV261-derived multicopy plasmid or in the chromosome

Strain, plasmid, or mutation	MIC ($\mu\text{g/ml}$) ^a		
	BDQ	CFZ	IMP
CIP104536 ^T (S)	0.06	0.5	8
Multicopy plasmid			
pMV261	0.125	0.5	8
pAtpE	0.125	0.5	8
pAtpE ^{D29V}	0.125	1	8
pAtpE ^{A64P}	0.125	1	8
Chromosomal mutation			
<i>atpE</i> _{D29V}	16	0.5	8
<i>atpE</i> _{A64P}	16	0.5	8

^aThe MIC was determined in cation-adjusted Mueller-Hinton broth. Data are representative of two independent experiments. BDQ, bedaquiline; CFZ, clofazimine; IMP, imipenem.

mutated versions (pAtpE^{D29V} and pAtpE^{A64P} carrying the D29V and A64P replacements, respectively) of the protein in *M. abscessus*. However, neither overproduction of the wild type nor of the D29V or A64P variants resulted in increased MIC values compared to the parental CIP104536^T strain or the control strain carrying the empty pMV261 (Table 2). This may be explained by the fact that in these transformed strains, overexpression of the different AtpE subunits is not sufficient to titrate the drug to the extent that higher drug concentrations are needed to inhibit the ATP synthase activity. Preiss et al. hypothesized that BDQ may block rotation of the c ring at the interface between the a subunit–c-ring interface of the ATP synthase F_o motor unit, implying that binding of BDQ to only one wild-type c subunit per complex can fully inhibit ion exchange and ATP synthesis activity (12). This hypothesis is supported by our observations that overexpression of mutated alleles in a wild-type background does not result in resistance. However, the definitive determination of a clinically relevant drug target requires the ability to transfer a single point mutation to a chromosomal gene that encodes the putative drug target and to demonstrate that this transfer is sufficient to confer drug resistance. Unexpectedly, when plating the various transformed strains densely ($\pm 10^8$ CFU) on agar plates supplemented with BDQ at 2 $\mu\text{g/ml}$, we observed an unusually high number of colonies with apparently acquired resistance in the case of the strains transformed with pAtpE^{D29V} or with pAtpE^{A64P}, but not with pAtpE (Fig. 4B). This was surprising given the lack of MIC upshift in liquid medium. We hypothesized that a recombinogenic event between the episomal copies of pAtpE^{D29V} (or pAtpE^{A64P}) and the chromosomal AtpE-encoding gene may have resulted in the introduction of a

FIG 4 Legend (Continued)

propagation on selective medium (kanamycin) maintains several copies of this plasmid in the cytosol. In step 3, after a few passages in liquid broth, homologous exchange between the wild-type chromosomal *atpE* gene and the mutated plasmid-borne alleles transfers the mutation onto the chromosomal allele. In step 4, in such a cell which has undergone homologous exchange, the plasmid now harboring the wild-type *atpE* allele is outnumbered by multiple copies of plasmid harboring the mutated allele, and reversion of the chromosomal *atpE* allele to wild-type is thus expected to occur at a low frequency. Plating the bacterial suspension on agar plates containing BDQ only allows the recombinant cells to form colonies. Sequencing analysis confirms that all BDQ-resistant colonies carry the mutations originally brought by the plasmid-borne *atpE* in the chromosomal *atpE*. In step 5, serial passage of bacteria in the absence of the selective antibiotic (kanamycin) cures the bacteria of the plasmid, resulting in the presence of only a mutated *atpE* allele in the chromosome. (D) PCR analysis of the chromosomal and plasmid-borne copy of *atpE* and sequencing verification of the D29V mutation in the different strains. Top (line drawing), open and closed arrows indicate where primers used in the PCR and sequencing analysis of chromosomal *atpE* and plasmid-borne *atpE*, respectively, bind. Middle (agarose gels), an open arrow indicates the band obtained for the chromosomal *atpE* locus (820 bp), and a solid arrow indicates the band obtained for the plasmid-borne *atpE* locus (386 bp). The same results were obtained regarding the A64P mutation (not shown). Bottom, representative chromatographs obtained during sequencing analysis of wild-type *atpE* alleles (left) and mutated *atpE* alleles (right). During sequencing analysis for the presence of the mutation, careful attention was paid to verify that the junctions between the *atpE* coding sequence and the sequences of chromosomal or plasmid origin surrounding it were indeed present in the sequence obtained from the chromosomal *atpE* and plasmid-borne *atpE* PCR products, respectively. (E) Susceptibility/resistance profiles of the various strains studied to antibiotics. Bacterial cultures were spotted on LB medium alone or supplemented with either 100 $\mu\text{g/ml}$ kanamycin or 2 $\mu\text{g/ml}$ BDQ. Plates were incubated for 3 days at 37°C.

TABLE 3 Frequency of BDQ resistance in strains carrying or not a plasmid with wild-type or mutated *atpE* alleles

Strain or plasmid	BDQ (2 $\mu\text{g/ml}$)	
	Expt 1	Expt 2
CIP104536 ^T (S)	$<1.1 \times 10^{-8}$	$<7.8 \times 10^{-9}$
pAtpE	$<4.7 \times 10^{-8}$	$<9.8 \times 10^{-8}$
pAtpE ^{D29V}	5.6×10^{-5}	9.4×10^{-5}
pAtpE ^{A64P}	3.5×10^{-5}	1.1×10^{-5}

single nucleotide polymorphism (SNP) leading to BDQ resistance, by way of a mechanism illustrated in Fig. 4C. To verify this assumption, the chromosomal *atpE* gene from 10 randomly selected colonies of each plate was PCR amplified and sequenced. This analysis revealed that, indeed, all resistant colonies had acquired an SNP conferring either a D29V or an A64P mutation. Remarkably, this genetic event occurred at a very high frequency in the presence of BDQ, since the acquisition of the SNP leading to the D29V and A64P mutations happened at a frequency of 5.6×10^{-5} to 9.4×10^{-5} and 1.1×10^{-5} to 3.5×10^{-5} , respectively (Table 3). In sharp contrast, no spontaneous resistant mutants in the strain harboring the episomal copy of pAtpE were selected at 2 $\mu\text{g/ml}$, even when almost 10^9 CFU were plated. To further explore whether the transfer of the SNP leading to the D29V and A64P mutations in the chromosomal *atpE* locus is sufficient to confer resistance to BDQ, the strains were cured of their pAtpE^{D29V} and pAtpE^{A64P} plasmids by subculturing the colonies in the absence of kanamycin and BDQ. PCR and sequencing analyses demonstrated that the plasmids were lost, and the resulting strains differed from the parental CIP104536^T strain only by one SNP in *atpE* (Fig. 4D). Curing was further confirmed by the absence of growth on kanamycin of the two resulting strains, designated CIP104536^T_{*atpE*::D29V} and CIP104536^T_{*atpE*::A64P} (Fig. 4E). Importantly, these isogenic strains, but not the CIP104536^T parental strain, grew in the presence of 2 $\mu\text{g/ml}$ BDQ (Fig. 4E). This high resistance profile was further confirmed by their MIC of 16 $\mu\text{g/ml}$, corresponding to more than 100 times the MIC of the parental strain (Table 2). In contrast, the MIC values of clofazimine or imipenem against CIP104536^T_{*atpE*::D29V} and CIP104536^T_{*atpE*::A64P} were unaffected, indicating that the transfer of these mutations in *atpE* conferred specific resistance to BDQ. Complementation of the CIP104536^T_{*atpE*::D29V} and CIP104536^T_{*atpE*::A64P} strains with a wild-type copy of *atpE* on the pAtpE plasmid partially restored sensitivity to BDQ, while transformation of these strains with pAtpE^{D29V} and pAtpE^{A64P}, respectively, did not (Fig. 4E), confirming BDQ sensitivity as a dominant phenotype in these merodiploid strains. Furthermore, BDQ did not induce ATP depletion in the CIP104536^T_{*atpE*::D29V} and CIP104536^T_{*atpE*::A64P} strains (Fig. 3C), thus confirming the BDQ resistance profile of the *atpE* mutant strains.

Overall, these results indicate that the single transfer of the D29V and A64P mutations into the chromosomal *atpE* locus is sufficient to confer high BDQ resistance, thus validating AtpE as the primary target of BDQ in *M. abscessus*.

DISCUSSION

Recognized as a cause of chronic pulmonary infections, especially in individuals with altered host defenses or disrupted airway clearance mechanisms, *M. abscessus* appears as a major infectious threat to the airway in CF patients, and reports suggest increased prevalence in recent years (27, 30). This situation is worsened by the fact that antibiotherapy against *M. abscessus* is often unsuccessful and/or poorly tolerated by patients. *M. abscessus* is notorious for being intrinsically resistant to most antibiotics (31), thus rendering these infections particularly complicated, difficult to treat, and associated with a high rate of therapeutic failure (32). Because of the absence of new active molecules, recent studies have focused on exploring the synergy of already-available drug combinations against *M. abscessus* (33) or on repurposing approved drugs (34, 35). In this study, we evaluated the *in vitro* and *in vivo* activity of BDQ against *M. abscessus*.

In agreement with a recent study addressing the activity of BDQ against several NTMs in China (36), we found that BDQ exhibited low MIC values against a collection of clinical isolates from France. The efficacy was not dependent on the bacterial morphology (rough versus smooth) and was equal in the different *M. abscessus* subspecies, which is of interest since *M. abscessus*, *M. massiliense*, and *M. bolletii* can respond differently to some antibiotics (37).

While two previous studies have evaluated the activity of BDQ against *M. abscessus* in immunocompromised mice, one conducted in GKO mice showing the efficacy of BDQ treatment in reducing bacterial loads (19) and another one in nude mice with no decrease in pulmonary loads (20), we report here a robust and sustained effect of BDQ in infected zebrafish. Our results highlight the *in vivo* efficacy of BDQ in this animal model, allowing visualizing in a dose- and time-dependent manner the dynamics of cord and abscess formation/resorption. BDQ exerted a very strong and positive impact on embryo survival (around 80% at 13 dpi). However, treatment with BDQ did not fully abrogate the development of infection cords, which in turn initiate the formation of infection foci (28). This may ultimately lead to the killing of the remaining 20% infected embryos observed at 13 dpi, which were not protected by the treatment. The notion of cords being associated with killing has recently been emphasized by a deletion mutant of *MAB_4780*, encoding a dehydratase, which exhibited a pronounced defect in cording, correlating with an extremely attenuated phenotype not only in wild-type but also in immunocompromised zebrafish (38). Overall, the present study reports the usefulness of zebrafish as a preclinical model to evaluate in real time the efficacy of BDQ against *M. abscessus* infection in the sole context of innate immunity. Since zebrafish embryos have been successfully used in the past to test the efficacy of the two clinically relevant drugs, clarithromycin and imipenem (22), and to demonstrate the potential of a combination consisting of a β -lactam (amoxicillin or imipenem) and a β -lactamase inhibitor (avibactam) (23, 24), future studies should address the *in vivo* efficacies of these drugs given in combination with BDQ using the zebrafish model of *M. abscessus* infection.

In vitro assessments of BDQ suggest that the drug exerts a bacteriostatic effect by targeting the F_0F_1 ATP synthase. This is in contrast to the reported bactericidal potency of BDQ in *M. tuberculosis* (9). However, similar to *M. abscessus*, it was reported earlier that inhibition of the ATP synthase of *M. avium* is also not bactericidal (39). It remains to be determined why BDQ is bacteriostatic against some NTM species despite being an excellent growth inhibitor at low doses *in vitro*. Nevertheless, this work indicates that oxidative phosphorylation is an attractive target space for future drug development in *M. abscessus*, as recently proposed in the case of *M. tuberculosis* (52, 53). A recent high-throughput screen in mycobacterial inverted membrane vesicles and subsequent biochemical deconvolution identified a squaramide series as a new class of ATP synthase inhibitors (40). The notion that these compounds may also inhibit the growth of *M. abscessus* is an attractive hypothesis, which requires further investigations. In addition, inhibitors of energy metabolism may prove highly suitable in drug combination regimens due to interference with drug efflux (53). Efflux pumps have emerged recently as important determinants in drug resistance mechanisms in *M. tuberculosis* (41, 42), as well as in *M. abscessus* (7). Because a sufficiently high proton motive force and ATP levels have to be maintained for drug extrusion mechanisms, draining the energy supply of efflux pumps may represent an alternative strategy to impede drug efflux. Since BDQ blocks oxidative phosphorylation, it may indirectly interfere with efflux pump function. Because TAC analogues are actively transported by an MmpL5-like efflux pump mechanism (7), the combination of these compounds with BDQ may lead to increased intracellular levels of the TAC analogues, thereby enhancing their potency against *M. abscessus*.

Another major finding of this study is the demonstration that the ATP synthase represents the primary target of BDQ in *M. abscessus*, thanks to the construction of genetically isogenic mutant strains. We report here a powerful method to introduce single point mutations into genes encoding potential drug targets. This recombinering strategy involves transformation of a BDQ-susceptible strain with a multicopy plasmid carrying *atpE* and harboring the desired mutation(s) to introduce these into the

chromosomal *atpE* locus. Maintaining the BDQ pressure allowed the selection of double homologous recombination events between the episomal and chromosomal *atpE* locus. Subsequent curing of the plasmid leads to an isogenic strain differing from the parental strain by only one SNP in *atpE*. The generation of the CIP10453^T_*atpE*::D29V and CIP104536^T_*atpE*::A64P isogenic mutant strains revealed that these amino acid substitutions led to high resistance to BDQ, probably because of the structural interference of these mutations with BDQ binding, as previously established by biochemical and X-ray crystallographic studies in *M. tuberculosis* (12). The genetic approach described here by introducing independently two single point mutations in AtpE (D29V and A64P) provides a proof of concept of a simple approach that could be applied to characterize other antimycobacterial drug targets and single point mutations and assessment of these consequences for antibiotic resistance in *M. abscessus*.

To gain further insights into the mechanisms of resistance to BDQ in *M. abscessus*, nine spontaneous resistant mutants exhibiting low levels of resistance at 4- to 8-fold the MIC to BDQ were selected and sequenced for the presence of eventual mutations in genes previously correlated with BDQ resistance in *M. tuberculosis* (data not shown). The genes sequenced included *atpE* (9, 10), *atpC* (13), and *pepQ* (43), as well as three genes encoding TetR repressors (*MAB_4384*, *MAB_4312*, and *MAB_4709c*) of MmpL5-like proteins (7). However, no SNPs were identified in any of these genes, suggesting the existence of additional mechanisms of resistance to BDQ in *M. abscessus*. Moreover, *M. abscessus* mutants that were highly resistant to TAC analogues (7) due to increased expression of the MmpL5-like MAB_4382c efflux system were not resistant to BDQ (data not shown). Although these results exclude MAB_4382c as an efflux pump in BDQ resistance, it remains possible that other MmpL members contribute to low BDQ resistance levels. This view is emphasized by the unusual abundance of the MmpL class of efflux pumps in the *M. abscessus* clade, compared to most other mycobacterial species, presumably contributing to the intrinsic resistance of *M. abscessus* to many antibiotics (44). Further investigations are needed to decipher whether drug efflux-mediated mechanisms participate in BDQ resistance, as proposed recently in BDQ-resistant *M. tuberculosis* strains through the upregulation of MmpL5 (45–47).

In summary, this work suggests that BDQ may have a place in new drug regimens for *M. abscessus* infections, especially in CF patients where current therapeutic outcomes are very poor. Since BDQ can be given orally, the use of this drug may therefore improve the *M. abscessus* treatment outcomes, particularly for clarithromycin-resistant *M. abscessus* lung diseases. Future studies are now required to identify new combinations of oxidative phosphorylation inhibitors for the design of completely new drug regimens against *M. abscessus*.

MATERIALS AND METHODS

Bacterial strains. *M. abscessus* subsp. *abscessus* CIP104536^T, *M. abscessus* subsp. *bolletii* CIP108541^T, and *M. abscessus* subsp. *massiliense* CIP108297^T reference strains and clinical isolates from CF and non-CF patients were reported previously (7, 33). Strains were routinely grown and maintained at 30°C in Middlebrook 7H9 broth (BD Difco) supplemented with 0.05% Tween 80 (Sigma-Aldrich) and 10% oleic acid-albumin-dextrose-catalase (OADC enrichment; BD Difco) (7H9^{T/OADC}) or on Middlebrook 7H10 agar (BD Difco) containing 10% OADC enrichment (7H10^{OADC}) and in the presence of antibiotics, when required. For drug susceptibility testing, bacteria were grown in cation-adjusted Mueller-Hinton broth (CaMHB; Sigma-Aldrich).

Drug susceptibility testing. The CLSI guidelines (48) were followed to determine the MICs based on the broth microdilution method in CaMHB using an inoculum containing 5×10^6 CFU/ml in the exponential-growth phase. Bacteria (100 μ l) were seeded in 96-well plates, and 2 μ l of drug at its highest concentration was added to the first wells containing double the volume of bacterial suspension (200 μ l). Twofold serial dilutions were then carried out, and incubation with drugs was performed at 30°C for 3 to 5 days. MICs were recorded by visual inspection and by absorbance at 560 nm to confirm visual recording. Experiments were done in triplicate on three independent occasions.

Time-kill assay. Microtiter plates were set up as for MIC determination. Serial dilutions of the bacterial suspension were plated after 0, 24, 48, 72, and 96 h of exposure to different drug concentrations. CFU were enumerated after 4 days of incubation at 30°C.

Zebrafish care and ethics statements. All zebrafish experiments were approved by the Direction Sanitaire et Vétérinaire de l'Hérault et Comité d'Éthique pour l'Expérimentation Animale de la Région Languedoc Roussillon under reference no. CEEA-LR-1145. Experiments were done using the *golden*

TABLE 4 Oligonucleotides used in this study

Primer ^a	Sequence ^b
atpE_Fw	GTAAGACCGCCGAACCTCTTG
atpE_Rev	TGGGGATGAGGAAGTTGTTC
pepQ_Fw	AAGCCATTGGGTTGTGAGAG
pepQ_Rev	GCGGTACAGGTAGGTGGTGT
MAB_4312_Fw	GTTATGGGCCGACAGTAGGA
MAB_4312_Rev	GATGTGCTTCGGGTTGAAAT
MAB_4384_Fw	TTCTGAGTTGGATGTACGGGCCGGATGA
MAB_4384_Rev	CTGCCACGAGATCGACGCCGCTGA
MAB_4709c_Fw	CTGGGTCCGAGTAGAAGCTG
MAB_4709c_Rev	AGATGCGAAGCGTTCCTTGAT
atpE_cloning_Fw	TGGCGGACCCACAATTGTTG
atpE_cloning_HindIII_Rev	GAGTGAAAGCTTTAGCTGGGCCGGGAGT
atpE_D29V_Fw	CCGGTATCGGTGTCGGTATCGCCGGTAA
atpE_D29V_Rev	TTACCGGCGATACCGACACCGATACCGG
atpE_A64P_Fw	GTCTGGTTGAGGCTCCGTACTTCATCAACCT
atpE_A64P_Rev	AGGTTGATGAAGTACGGAGCCTCAACCAGAC
pMV5'	CGCCCGGCCAGCGTAAGTAGC
pMV3'	GCCTGGCAGTCGATCGTACG

^aFw, forward; Rev, reverse.

^bRestriction sites are underlined, and mutated residues are in bold type.

mutant (49) crossed with wild-type AB zebrafish, maintained as described earlier (28). The ages of the embryos are expressed in hours postfertilization (hpf).

Assessment of BDQ efficacy in infected zebrafish. Rough *M. abscessus* CIP104536^T (ATCC 19977^T) carrying pTEC27 (plasmid 30182; Addgene) and expressing the red fluorescent protein tdTomato was prepared and microinjected in zebrafish embryos, according to procedures described earlier (28, 29). Briefly, mid-log-phase cultures of *M. abscessus* expressing tdTomato were centrifuged, washed, and resuspended in phosphate-buffered saline (PBS) supplemented with 0.05% Tween 80 (PBS-T). Bacterial suspensions were then homogenized through a 26-gauge needle and sonicated, and the remaining clumps were allowed to settle down for 5 to 10 min. Bacteria were concentrated to an optical density at 600 nm (OD₆₀₀) of 1 in PBS-T and injected intravenously (≈2 to 5 nl containing 50 to 300 CFU) into the caudal vein in 30-h-postfertilization (hpf) embryos previously dechorionated and anesthetized. To follow infection kinetics and embryo survival, infected larvae were transferred into 24-well plates (2 embryos/well) and incubated at 28.5°C. The CFU numbers in the inoculum were determined by injection of 2 nl of the bacterial suspension in sterile PBS-T and plating on 7H10 with 500 μg/ml hygromycin.

Drugs were added at 1 day postinfection (dpi) directly into the water containing the embryos. Three doses were tested, corresponding to 1, 2, and 3 μg/ml BDQ. *In vivo* drug efficacy was determined by following the survival of embryos as well as by monitoring the evolution of the abscesses and cords within whole embryos under the microscope (22). Survival curves were determined by recording dead embryos (no heartbeat) every day for up to 13 days.

Microscopy and image analysis. In order to visualize the infection foci, abscesses, and cords, infected larvae were anesthetized with tricaine, positioned on 35-mm dishes, and then immobilized in 1% low-melting-point agarose and covered with water containing tricaine. Bright-field and fluorescence pictures of live infected embryos were taken with a Zeiss microscope equipped with a Zeiss Plan Neofluar Z 1×/0.25 FWD objective and an Axiocam503 monochrome (Zeiss) camera, with acquisition and processing using ZEN 2 (blue edition). The final image analysis was carried out using GIMP 2.6 to merge fluorescent and bright-field images and to adjust levels and brightness and to remove out-of-focus background fluorescence.

Selection of spontaneous resistant mutants. Exponentially growing *M. abscessus* cultures were plated on 7H10^{OADC} containing 0.5 and 1 μg/ml BDQ, corresponding to 4× and 8× the MIC, respectively. After 1 week of incubation at 30°C, single colonies were selected and grown in liquid medium and individually subjected to MIC determination and scored for resistance to BDQ.

DNA constructs and homologous recombination. All oligonucleotides used in this study are listed in Table 4. For cloning, PCR amplifications were performed using purified *M. abscessus* genomic DNA and Phusion polymerase (Finnzymes, Finland). The *atpE* coding sequence was PCR amplified using the primers atpE_cloning_Fw and atpE_cloning_HindIII_Rev, and the product was digested with HindIII and ligated to MscI-HindIII-linearized pMV261, yielding pAtpE. To introduce D29V and A64P mutations in *atpE*, the overlap extension PCR method was followed (50). Briefly, the primers atpE_cloning_Fw and atpE_D29V_Rev and pAtpE as the template were first used to generate a PCR product. In parallel, the primers atpE_D29V_Fw and atpE_cloning_Rev and pAtpE were used to generate a second PCR product. Subsequently, the two PCR products were mixed in a single fresh Phusion PCR lacking any primers and cycled once with the following conditions: 40 s at 98°C, 10 min at 60°C, and 2 min at 72°C. The primers atpE_cloning_Fw and atpE_cloning_HindIII_Rev were then added, and a standard Phusion PCR cycling protocol was executed. The product was digested with HindIII and ligated to MscI-HindIII-linearized pMV261 to produce pAtpE^{D29V}. The same procedure was followed to generate pAtpE^{A64P} using the appropriate mutagenic primers. For sequencing reactions of spontaneous BDQ-resistant strains, includ-

ing the strains CIP104536^T::*atpE*^{D29V} and CIP104536^T::*atpE*^{A64P}, PCRs were performed using the GoldStar ready-to-use PCR mix (Eurogentec, France), using the primers listed in Table 4 and 1 μ l of a small aliquot of boiled bacterial culture as the template.

Intracellular ATP quantification. Intracellular ATP levels were determined using a 96-well flat-bottom plate, as described previously for *M. tuberculosis* (51). *M. abscessus* was exposed to BDQ or amikacin (negative control) and incubated for 180 min at 32°C. Twenty-five microliters of *M. abscessus* culture was mixed with an equal volume of the BacTiter-Glo reagent in 96-well flat-bottom white plates and incubated for 5 min in the darkness. Luminescence was detected using a BioTek Cytation 3 multimode reader, and the values obtained were plotted using GraphPad Prism 6 software.

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