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SUSCEPTIBILITY

In Vitro Activity of Bedaquiline against Nontuberculous Mycobacteria in China

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ABSTRACT The main goal of our study was to evaluate the *in vitro* bedaquiline susceptibility of six prevalent species of pathogenic nontuberculous mycobacteria (NTM) in China. In addition, we investigated the potential molecular mechanisms contributing to bedaquiline resistance in the different NTM species. Among slowly growing mycobacteria (SGM), bedaquiline exhibited the highest activity against *Mycobacterium avium*; the MIC_{50} and MIC_{90} values were 0.03 and 16 mg/liter, respectively. Among rapidly growing mycobacteria (RGM), *Mycobacterium abscessus* subsp. *abscessus* (*M. abscessus*) and *Mycobacterium abscessus* subsp. *massiliense* (*M. massiliense*) seemed more susceptible to bedaquiline than *Mycobacterium fortuitum*, with MIC_{50} and MIC_{90} values of 0.13 and >16 mg/liter, respectively, for both species. On the basis of bimodal distributions of bedaquiline MICs, we proposed the following epidemiological cutoff (ECOFF) values: 1.0 mg/liter for SGM and 2.0 mg/liter for RGM. Among *M. avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *M. abscessus*, *M. massiliense*, and *M. fortuitum* isolates, 14 (29.8%), 41 (27.2%), 33 (39.3%), 44 (20.2%), 42 (25.8%), and 7 (31.8%), respectively, were resistant to bedaquiline. No significant differences in the proportions of bedaquiline resistance among these species were observed ($P > 0.05$). Genetic mutations were observed in 74 isolates (10.8%), with all nucleotide substitutions being synonymous. In conclusion, our data demonstrate that bedaquiline shows moderate *in vitro* activity against NTM species. Using the proposed ECOFF values, we could distinguish between bedaquiline-resistant and -susceptible strains with the broth dilution method. In addition, no nonsynonymous mutations in the *atpE* gene that conferred bedaquiline resistance in all six NTM species were identified.

KEYWORDS nontuberculous mycobacteria, bedaquiline, ECOFF

Nontuberculous mycobacteria (NTM) are a group of all *Mycobacterium* species with the exception of the obligate *Mycobacterium tuberculosis* complex and *Mycobacterium leprae* (1). Although NTM are considered typically environmental organisms, NTM infections have attracted more attention due to their increased prevalence worldwide in the past 2 decades (2, 3). In many high-income countries, NTM disease is a significant contributor to morbidity and death among immunocompromised individuals (4). The major obstacle to addressing NTM disease is associated with its natural resistance to antibacterial drugs, resulting in disappointing clinical outcomes with the currently available treatment regimens (5). Therefore, there is an urgent need to develop and to employ novel and more effective antibiotics for the treatment of NTM infections (6, 7).

Bedaquiline is a novel diarylquinoline antibiotic, which exhibits potent activity against mycobacteria by inhibiting ATP synthase (8). On the basis of favorable results in a number of preclinical and clinical trials, this drug was approved in 2012

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by the U.S. FDA for use in the treatment of multidrug-resistant (MDR) tuberculosis (TB) (9). In addition, bedaquiline has been shown to have *in vitro* bacteriostatic activity against a wide range of NTM isolates (10). A recent preliminary report demonstrated potential clinical and microbiological activity of bedaquiline in patients with NTM disease. Taken together, the previous findings indicate promising prospects for the use of bedaquiline as part of combination therapy to treat NTM disease (4).

Prior to the application of bedaquiline, reliable results of *in vitro* antimicrobial susceptibility testing are urgently needed to guide the use of bedaquiline in the treatment of mycobacterial infections (6). Previous studies have recommended that the MIC breakpoint for the use of bedaquiline be 0.25 mg/liter for *Mycobacterium tuberculosis* (11), whereas very little attention has been paid to *in vitro* susceptibility profiles for bedaquiline against NTM. Therefore, data regarding the MIC distributions of different NTM species are essential for formulating practical recommendations regarding the use of bedaquiline for the treatment of infections due to different NTM species. The main goal of our study was to evaluate the *in vitro* susceptibility to bedaquiline of six prevalent mycobacterial species associated with NTM disease in China. Based on these distributions, the epidemiological cutoff (ECOFF) values for bedaquiline were proposed for these NTM species. In addition, we investigated the potential molecular mechanism contributing to the bedaquiline resistance in these different NTM species.

RESULTS

Bedaquiline MICs for NTM isolates. A total of 685 NTM isolates were included in this study, including 47 *Mycobacterium avium* (6.9%), 151 *Mycobacterium intracellulare* (22.0%), 84 *Mycobacterium kansasii* (12.3%), 218 *Mycobacterium abscessus* subsp. *abscessus* (*M. abscessus*) (31.8%), 163 *Mycobacterium abscessus* subsp. *massiliense* (*M. massiliense*) (23.8%), and 22 *Mycobacterium fortuitum* (3.2%) isolates. The bedaquiline MICs for the NTM isolates are summarized in Table 1. Among slowly growing mycobacteria (SGM), bedaquiline exhibited the highest activity against *M. avium*; the MIC_{50} and MIC_{90} were 0.03 and 16 mg/liter, respectively. Among rapidly growing mycobacteria (RGM), *M. abscessus* and *M. massiliense* seemed more susceptible to bedaquiline than *M. fortuitum*, with MIC_{50} and MIC_{90} values of 0.13 and >16 mg/liter, respectively, for both species.

ECOFF values for NTM isolates. As shown in Fig. 1 and 2, the bedaquiline MIC values of six NTM species followed bimodal distributions. Most of the tested isolates showed MIC values of less than 0.016 mg/liter and greater than 8 mg/liter. Based on the guidelines for determining ECOFF values, we propose the following ECOFF values: 1.0 mg/liter for SGM and 2.0 mg/liter for RGM. When 1.0 mg/liter was used as the cutoff value, 14/47 *M. avium* isolates (29.8%), 41/151 *M. intracellulare* isolates (27.2%), and 33/84 *M. kansasii* isolates (39.3%) were resistant to bedaquiline. Statistical analysis revealed that the proportions of bedaquiline-resistant isolates showed no significant differences among these species ($P > 0.05$). For RGM, resistance to bedaquiline was noted for 20.2% of *M. abscessus* isolates (44/218 isolates), 25.8% of *M. massiliense* isolates (42/163 isolates), and 31.8% of *M. fortuitum* isolates (7/22 isolates). Similar to SGM, no significant differences in the percentages of bedaquiline-resistant isolates were observed among RGM.

Mutations in *atpE* genes. The entire *atpE* genes of 685 NTM isolates were sequenced. The DNA sequence chromatogram found that genetic mutations were observed in 74 (10.8%) of those 685 isolates; all of those nucleotide substitutions were synonymous mutations, resulting in no amino acid change. As shown in Table 2, *M. fortuitum* had the highest frequency of genetic mutations (22.7% [5/22 isolates]), followed by *M. massiliense* (12.8% [21/163 isolates]), *M. avium* (10.6% [5/47 isolates]), *M. intracellulare* (10.6% [16/151 isolates]), *M. abscessus* (9.6% [21/218 isolates]), and *M. kansasii* (7.1% [6/84 isolates]). We further analyzed the relationship between nucleotide substitutions and bedaquiline MIC values. The

TABLE 1 Distribution of bedaquiline MIC values for NTM isolates enrolled in this study

Classification and species[a]	No. of strains with MIC of:												Total no. of strains	MIC_{50} (mg/liter)	MIC_{90} (mg/liter)	Proportion of resistant strains (%)
	0.016 mg/liter	0.031 mg/liter	0.062 mg/liter	0.12 mg/liter	0.25 mg/liter	0.5 mg/liter	1 mg/liter	2 mg/liter	4 mg/liter	8 mg/liter	16 mg/liter	>16 mg/liter				
SGM																
M. avium	23	5	2	1	1	1	0	1	2	5	3	3	47	0.03	16	29.8
M. intracellulare	68	16	10	8	5	2	1	1	3	4	12	21	151	0.03	>16	27.2
M. kansasii	27	8	8	4	2	1	1	1	3	5	8	16	84	0.06	>16	39.3
RGM																
M. abscessus	6	19	51	53	24	15	4	2	3	5	7	29	218	0.13	>16	20.2
M. massiliense	5	18	37	29	14	10	5	3	2	2	6	32	163	0.13	>16	25.8
M. fortuitum	1	2	4	3	2	2	1	0	1	1	2	3	22	0.25	>16	31.8

[a]SGM, slowly growing mycobacteria; RGM, rapidly growing mycobacteria; MIC_{50}, concentration required to inhibit the growth of 50% of the strains; MIC_{90}, concentration required to inhibit the growth of 90% of the strains.

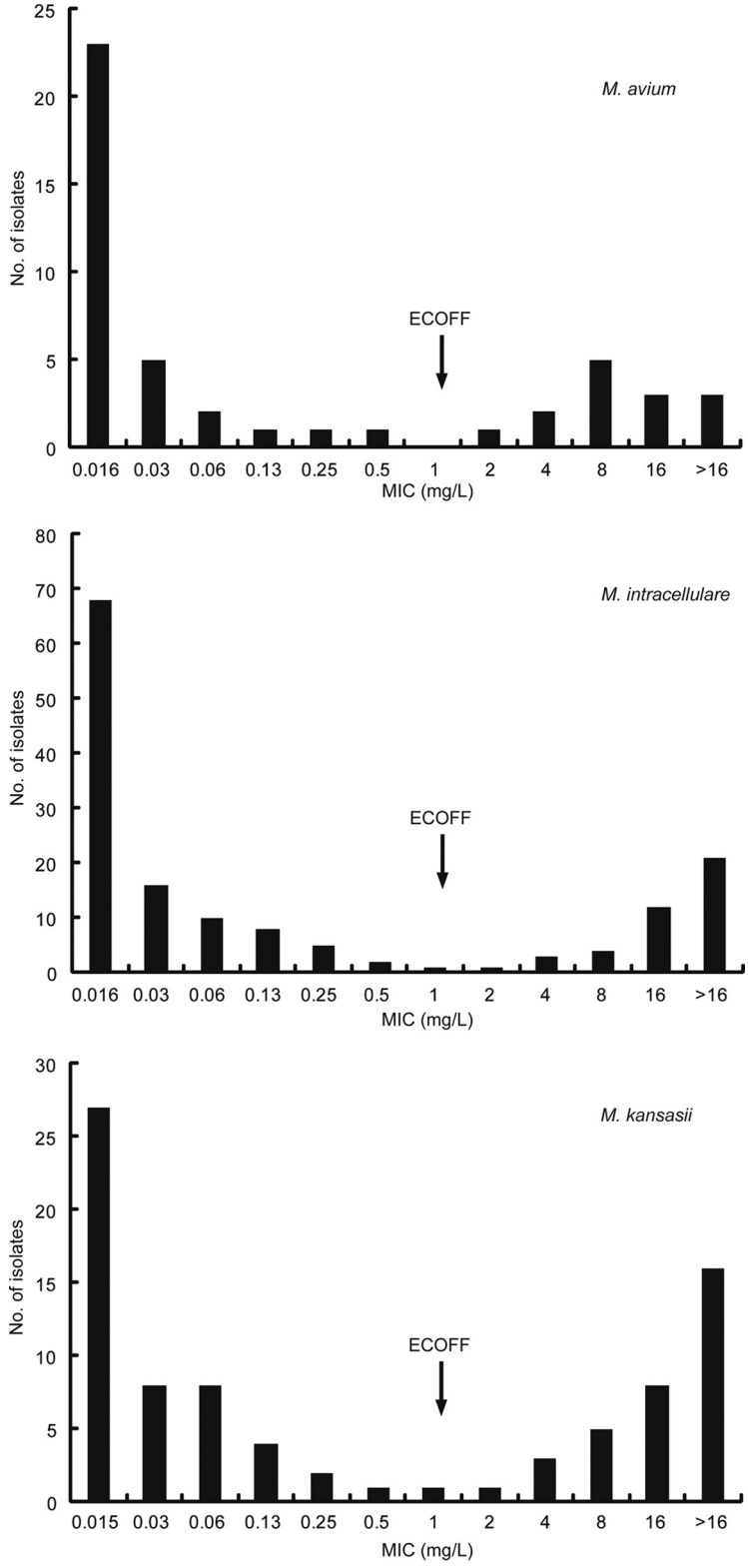

FIG 1 Distribution of MIC values for slowly growing mycobacterial strains. The arrows represent the proposed ECOFF value for slowly growing mycobacteria.

majority of isolates harboring mutations (91.9% [68/74 isolates]) were susceptible to bedaquiline, whereas only six isolates (8.1% [6/74 isolates]) showed resistance to bedaquiline, indicating that these synonymous nucleotide polymorphisms may not be associated with bedaquiline resistance.

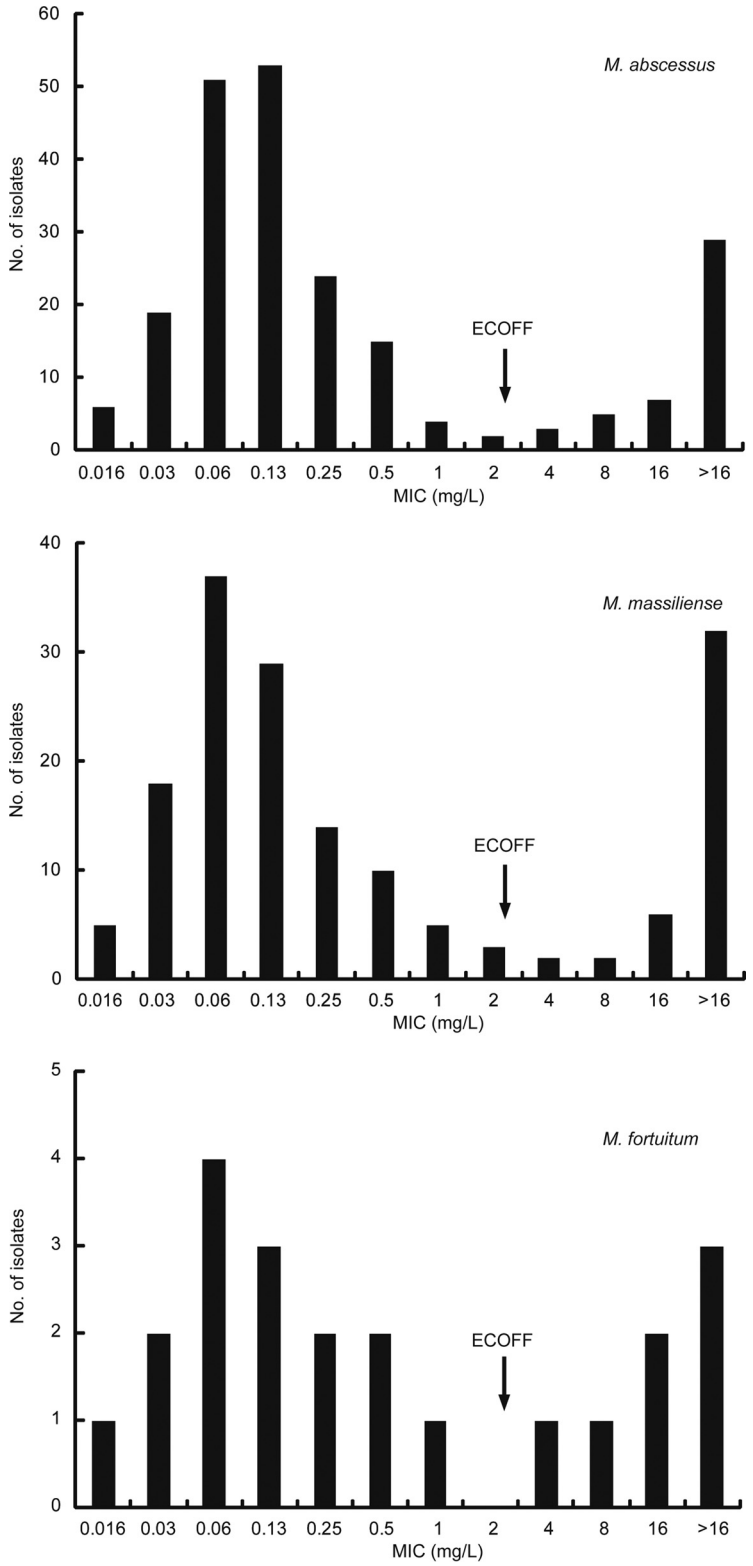

FIG 2 Distribution of MIC values for rapidly growing mycobacterial strains. The arrows represent the proposed ECOFF value for rapidly growing mycobacteria.

DISCUSSION

Due to the intrinsic resistance to most available antibiotics, the nontuberculous mycobacteria pose a unique challenge for clinical treatment (12). There is interest in evaluating new anti-TB compounds against NTM, which will provide new options for

TABLE 2 Distribution of MIC values for NTM isolates harboring mutations in the *atpE* gene

Species	Mutation in *atpE* gene		No. of strains with MIC of:												
	Nucleotide change	Amino acid change	0.016 mg/liter	0.031 mg/liter	0.062 mg/liter	0.12 mg/liter	0.25 mg/liter	0.50 mg/liter	1 mg/liter	2 mg/liter	4 mg/liter	8 mg/liter	16 mg/liter	>16 mg/liter	Total no. of strains
M. avium	C186T	Val62Val		1		1								1	3
	C186A	Val62Val			1	1									2
M. intracellulare	C147T	Gly49Gly	6	2	3	2			1		1			1	16
M. kansasii	T186C	Gly62Gly		2	3				1						6
M. abscessus	T72C	Gly24Gly			3	1		3							7
	T105C	Ala35Ala		2	5	4	2				1				14
M. massiliense	T72C	Gly24Gly			3	7	4	3						1	18
	T105C	Ala35Ala		1		1				1					3
M. fortuitum	A195G	Glu65Glu			2		1		1				1		5

treatment of NTM infections (12). In this study, we first evaluated the *in vitro* efficacy of bedaquiline with a large number of clinical NTM isolates. Although a bedaquiline breakpoint was established for *M. tuberculosis* (11), there were no clear criteria regarding whether a given NTM isolate is susceptible to bedaquiline. Hence, the most important finding of this study is the proposal of ECOFF values, i.e., 1.0 mg/liter for SGM and 2.0 mg/liter for RGM. On the basis of the ECOFF values, our data demonstrate that bedaquiline exhibits moderate activity against various NTM species. For *M. avium*, the proportion of bedaquiline-resistant isolates was 29.8%, which is greater than the values for clarithromycin (3.0%), amikacin (9.2%), and moxifloxacin (10.8%) but lower than those for rifampin (RIF) (38.5%), linezolid (40.0%), and ethambutol (EMB) (40.0%) (13). Similar to the findings for *M. avium*, the proportion of bedaquiline-resistant strains was smaller than the values for RIF (66.0%) and EMB (49.5%) for *M. intracellulare* in China (13). Clarithromycin in combination with RIF and EMB is the cornerstone of the treatment of *M. avium* complex (MAC) lung disease (6). In view of the high-level resistance to RIF and EMB of MAC strains in China, bedaquiline may provide an alternative to generate an effective regimen for MAC infections. In addition, a recent report on *in vitro* bedaquiline susceptibility testing of MAC strains by Brown-Elliott and colleagues revealed that 50% of the MAC isolates had MIC_{50} values of $\leq$0.008 mg/liter (14), which is significantly lower than the MIC_{50} of 0.03 mg/liter from the current study. Although the exact reasons remain unknown, several potential reasons may be responsible for the discrepancy with respect to other studies. In China, due to the lack of the capability to identify mycobacterial species, a larger proportion of NTM cases may be misdiagnosed as MDR-TB, resulting in potential exposure to second-line anti-TB drugs, including clofazimine. In view of the cross-resistance between clofazimine and bedaquiline (15, 16), preliminary exposure to clofazimine may be an important contributor to the significant difference in MICs. Alternatively, the high MIC values for bedaquiline against MAC strains may be due to the abuse of antibiotics in the animal and food industries, which is associated with high concentrations of antibiotics in the environment (17). Because they are opportunistic pathogens, overexposure to broad-spectrum antibiotics in the natural habitat may accelerate the emergence of intrinsically drug-resistant MAC strains by decreasing cell permeability, which also may be a potential reason for the different bimodal MIC distribution profiles of MAC strains.

M. abscessus infections are associated with the lowest cure rate among various NTM species, which is largely due to the emergence of inducible macrolide resistance in *M. abscessus* (12). As a consequence, the treatment of clarithromycin-resistant *M. abscessus* relies on the use of amikacin and cefoxitin (12, 18), although a recent study from China reported that 32% and 55% of *M. abscessus* isolates were resistant and intermediate to cefoxitin, respectively (19). Considering the large proportion of cefoxitin-resistant isolates, the use of bedaquiline is more likely to be a promising choice for *M. abscessus* infections in China. Consistent with our findings, a small preliminary report by Philley and colleagues revealed that bedaquiline produced potential clinical and microbiological activity in patients with advanced MAC or *M. abscessus* disease (4). In contrast, nude mouse model experiments demonstrated that bedaquiline did not prevent death when used alone, which might be associated with high minimal bactericidal concentrations (18). Despite the conflicting observations from different studies, our *in vitro* susceptibility data indicate that the addition of bedaquiline to a preferred drug combination may serve as a starting point for the optimized use of this novel anti-TB compound against *M. abscessus*. Large clinical trials are urgently needed to confirm the efficacy of bedaquiline in the management of *M. abscessus* and other NTM lung diseases.

Resistance to bedaquiline is associated with genetic mutations in the *atpE* gene in *M. tuberculosis*, which encodes subunit c of the F0 subunit of ATP synthase (the target of bedaquiline). Numerous reports have found nucleotide substitutions in selected bedaquiline-resistant mutants, such as A63P and I66M in *M. tuberculosis* (20–22). Similar to those findings in *M. tuberculosis*, a recent study by Alexander et al. indicated that one nonsynonymous mutation in the *atpE* gene was associated with a 50-fold increase in the bedaquiline MIC in *M. intracellulare* (23). However, no nonsynonymous mutations in

the *atpE* gene that conferred bedaquiline resistance in all six NTM species were identified in our study. In a study by Huitric et al., only 15 (28.3%) of 53 bedaquiline-resistant *M. tuberculosis* isolates harbored mutations in *atpE* (24). The unsatisfactory correlation between *in vitro* susceptibility and genotypes in mycobacteria indicates that alternative resistance mechanisms must be involved in bedaquiline resistance. Several potential mechanisms conferring bedaquiline resistance in *M. tuberculosis* have been reported (15, 25). Milano and colleagues found that mutations in Rv0678, which encodes a regulatory protein of the MmpS5-MmpL5 efflux system, were associated with bedaquiline and clofazimine cross-resistance in MDR-TB patients receiving bedaquiline treatment (25). Another gene, i.e., *pepQ*, encoding a putative Xaa-Pro aminopeptidase, has also been determined to confer low-level resistance to bedaquiline in *M. tuberculosis* (15). The efflux pump and other natural mechanisms doubtless result in low-level resistance, while high-level drug resistance is attributed to mutations in the target genes (26). Hence, we hypothesize that bedaquiline must engage targets other than *atpE* to achieve its bacteriostatic activity.

This report has several limitations. First, all of the experiments in this study were carried out *in vitro* with clinical NTM isolates. Future studies are needed to determine the correlation between *in vitro* susceptibility and treatment outcomes in clinical trials. Second, some NTM isolates in this study had MICs of $<$0.016 mg/liter, which were not covered by our experimental system; this may hinder us in determining their true MICs. Third, sequencing of the *atpE* gene alone, and not Rv0678 and *pepQ*, was included in our study. Further analysis of the latter two genes will extend our knowledge of the molecular mechanisms conferring bedaquiline resistance in NTM species. Fourth, there is strong evidence that bedaquiline exhibits cross-resistance with clofazimine in *M. tuberculosis* (15, 16), although the cross-resistance profiles of these two compounds were not evaluated in the final analysis. Nevertheless, our observations provide important insights into the clinical application of bedaquiline for the treatment of NTM infections.

In conclusion, our data demonstrate that bedaquiline shows moderate *in vitro* activity against NTM species. Using ECOFF values of 1.0 mg/liter for SGM and 2.0 mg/liter for RGM, we could distinguish between bedaquiline-resistant and -susceptible strains by using the broth dilution method. In addition, no nonsynonymous mutations in the *atpE* gene that conferred bedaquiline resistance in all six NTM species were identified. Further studies are urgently needed to investigate the molecular mechanisms conferring bedaquiline resistance in NTM species.

MATERIALS AND METHODS

Ethics statement. The protocols used in this study were approved by the Ethics Committee of the Chinese Center for Disease Control and Prevention.

Bacterial strains. The strains used in this study, representing different geographical origins, were collected between 2011 and 2015 from Guangdong Chest Hospital, Shanghai Pulmonary Hospital, Lianyungang Fourth Hospital, Chongqing Yongchuan Hospital (affiliated with Chongqing Medical University), Inner Mongolia Fourth Hospital, and Kaifeng Pulmonary Hospital. All of the strains were identified as NTM species using multilocus sequence analysis, including 16S rRNA, *hsp65*, *rpoB*, and a 16S-23S rRNA internal transcribed spacer (ITS) sequence (13). The most prevalent NTM isolates associated with NTM diseases, including *M. avium*, *M. intracellulare*, *M. abscessu*s, *M. massiliense*, *M. kansasii*, and *M. fortuitum*, were included, whereas the other rare subspecies belonging to the *M. avium* complex, *M. abscessus* complex, and *M. fortuitum* complex were excluded from the current study.

MIC assays. Pure bedaquiline powder was a gift from Johnson & Johnson (Beerse, Belgium). To determine the bedaquiline susceptibility of NTM strains, broth microdilution assays were performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (27). Cation-adjusted Mueller-Hinton broth (CAMHB) enriched with oleic acid-albumin-dextrose-catalase (OADC) was used for SGM, while CAMHB without OADC was used for RGM. The bacterial suspensions were prepared from subcultures collected from 4-week-old cultures in Löwenstein-Jensen medium. The broth microdulution format was set up with 2-fold dilutions, and the concentrations of bedaquiline ranged from 0.016 to 16 mg/liter. Briefly, a suspension was prepared at a 0.5 McFarland standard, diluted, and inoculated into 96-well microtiter plates to achieve final organism concentrations of 10^5 cells/ml for both SGM and RGM. All plates were incubated at 37°C for 7 days for SGM and 3 days for RGM. All experiments were performed in triplicate. The MIC was defined as the lowest concentration that inhibited visible growth. The ECOFFs were determined according to the distribution profiles of MIC values. For unimodal MIC distributions,

ECOFFs were defined as concentrations representing ≥99.9% of the bacterial population; for bimodal MIC distributions, ECOFFs were set between the two populations (28).

DNA amplification and sequencing. The *atpE* gene encodes subunit c of the F0 subunit of ATP synthase, which is the target of bedaquiline (8). In this study, the *atpE* genes from different NTM species were analyzed by Sanger sequencing. DNA fragments were amplified with the primers listed in Table S1 in the supplemental material. PCR was performed in a final volume of 50 μl, containing 5 μl 10× PCR buffer, 200 μM each deoxynucleoside triphosphate (dNTP), 0.2 μM each primer set, and 1 U HotStar *Taq* polymerase (Qiagen). PCR was performed as follows: initial denaturation of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C; and final extension of 10 min at 72°C. The amplification products were sent to Tsingke Co. (Beijing, China) for DNA sequencing. DNA sequences were aligned with the homologous sequences of the reference mycobacterial strains by using BioEdit Sequence Alignment Editor 7.1.3 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Statistical analysis. The chi-square test was performed to compare the proportions of bedaquiline-resistant isolates between different NTM species, using SPSS 14.0 (SPSS Inc., Chicago, IL). Differences were considered significant if the *P* values were <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02627-16.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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