In Vitro Activity of Clofazimine against Nontuberculous Mycobacteria Isolated in Beijing, China

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ABSTRACT Due to the natural resistance of nontuberculous mycobacteria (NTM) to many antibiotics, the treatment of diseases caused by NTM is often long-term but unsuccessful. The main goal of this study was to evaluate the in vitro susceptibilities to clofazimine of 209 isolates consisting of different NTM species isolated in Beijing, China. Furthermore, 47 reference strains were also tested, including 30 rapidly growing mycobacterium (RGM) species and 17 slowly growing mycobacterium (SGM) species. The potential molecular mechanism contributing to clofazimine resistance of NTM was investigated as well. Clofazimine exhibited excellent activity against both reference strains and clinical isolates of different SGM species, and most of the strains had MICs far below 1 μg/ml. Although the majority of the clinical isolates of Mycobacterium abscessus and Mycobacterium fortuitum had MICs higher than 2 μg/ml, 17 out of the 30 reference strains of different RGM species had MICs below 1 μg/ml in vitro. According to the MIC distributions, the tentative epidemiological cut-off (ECOFF) values for Mycobacterium kansasii, Mycobacterium avium, and Mycobacterium intracellulare were defined at 0.5 μg/ml, 1 μg/ml, and 2 μg/ml, respectively. Intriguingly, single-direction cross-resistance between bedaquiline- and clofazimine (Cfz)-resistant isolates was observed among the tested NTM species. This study demonstrates that clofazimine had strong activity against most SGM species in vitro, as well as some RGM species. The data provide important insights into the possible clinical application of Cfz to treat NTM infections.

KEYWORDS cross-resistance, bedaquiline, clofazimine, nontuberculous mycobacteria, susceptibility

Nontuberculous mycobacteria (NTM) exist ubiquitously in water, food, soil, and dust (1). Despite being considered environmental organisms, the incidence and prevalence of NTM disease have increased globally in the past few decades (2–4). Treatment of diseases caused by NTM is very challenging, mainly due to the natural resistance of NTM to most antibiotics. Although regimens targeting the most prevalent NTM species have been recommended by some professional institutions, those recommendations are usually weakly evidence-based, and the treatment outcomes are generally poor. Hence, identifying highly active anti-NTM agents is still a priority for potent regimen establishment.

Clofazimine (Cfz), the prototype of the antibiotic rimino-phenazine, has been used for leprosy treatment since the 1950s. Although the exact mechanism of Cfz-mediated antimicrobial activity remains to be deciphered, the outer membrane appears to be the primary target of action (5, 6). In recent years, the deficiency of drugs against multidrug-resistant tuberculosis (MDR-TB) aroused interest in Cfz, and promising treatment outcomes have been achieved (7). Moreover, a few studies demonstrated that Cfz harbored strong inhibitory activity against clinical isolates of some slowly growing...
mycobacteria (SGM) in vitro (8–10), while the efficacy of Cfz against rapidly growing mycobacteria (RGM) remains controversial (8, 11). An understanding of the activity of Cfz against different NTM species is far from sufficient. Because of limited studies on drug susceptibility testing (DST) for Cfz, well-recognized and trustable Cfz susceptibility testing methods have not been established. Since the breakpoint for Cfz susceptibility testing is still uncertain, the cutoff values adopted by previous studies have been different, ranging from 0.8 μg/ml to 2 μg/ml (8, 12, 13), which makes the data from different studies incomparable.

The main goal of this study was to evaluate the in vitro activity of Cfz against NTM isolates collected in Beijing, China, and define the tentative epidemiological cutoff (ECOFF) values for the most prevalent NTM species. Furthermore, we investigated the potential molecular mechanism of Cfz resistance and studied the cross-resistance between Cfz and bedaquiline among different NTM species.

RESULTS

MICs and ECOFF of Cfz against NTM strains. The MICs of the 47 reference strains against Cfz are presented in Table 1. Cfz exhibited diverse activity against the recruited RGM species. Seventeen out of the 30 RGM species had MICs lower than 1 μg/ml, whereas other three RGM species had MICs of ≥4 μg/ml. Nevertheless, Cfz demonstrated uniform strong inhibitory activity against almost all of the tested SGM species, with MICs far below 1 μg/ml, except Mycobacterium triviale (MIC, ≥8 μg/ml).

The MIC distributions for the six most prevalent NTM species in this study are detailed in Fig. 1. Among the recruited SGM species, Cfz exhibited the strongest activity against Mycobacterium kansasii, with a tentative ECOFF at 0.5 μg/ml, and the overwhelming majority of isolates had MICs below 0.003 μg/ml. Cfz also demonstrated strong activity against Mycobacterium avium and Mycobacterium intracellulare isolates, with tentative ECOFFs at 1 μg/ml and 2 μg/ml, respectively. The activity of Cfz against Mycobacterium gordonae isolates was very different. While eight isolates had MICs lower than 0.003 μg/ml, another three isolates had MICs of ≥1 μg/ml. Due to the limited number of isolates, the ECOFF of M. gordonae was not determined. Among the recruited RGM species, Cfz harbored weak activity against Mycobacterium abscessus and Mycobacterium fortuitum, with MIC90 values of >8 μg/ml for both species. No ECOFF was determined for these two species due to the weak inhibitory activity of Cfz and the truncated MIC ranges. In addition, the susceptibility testing outcomes for species with ≤5 isolates are recorded in Table 2. Cfz demonstrated strong activity against some very pathogenic Mycobacterium species, such as M. szulgai, M. xenopi, and M. malmoense, and it is notable that all of them are SGM species. Three Mycobacterium chelonae isolates had MICs of 0.25, 0.5, and 8 μg/ml, demonstrating large MIC variance within species.

Alignment and mutation of Rv0678 homologues. A multiple-amino-acid-alignment outcome profile for the products of Rv0678 homologues of different mycobacterial species and the topologies of the proteins are shown in Fig. 2. The alignment outcome suggested that the homologues contain three conserved amino acids, Arg82, Asp88, and Arg90, which located within the DNA-binding domains of the proteins and are likely important for protein-DNA interactions.

In order to determine the relationship between mutations in Rv0678 homologous genes and Cfz resistance, the full-length Rv0678 homologues of M. avium and M. intracellulare were sequenced. Among the 22 M. avium isolates, no specific mutation within the Rv0678 homologues associated with Cfz resistance was identified. An Asp127Asn mutation was observed in both Cfz-susceptible and -resistant isolates. Among the 35 M. intracellulare isolates, an Asp92Glu mutation located in the DNA-binding domain was only found in one isolate, with an MIC of >8 μg/ml. Furthermore, an Ala153Pro mutation was detected in one M. intracellulare isolate, with an MIC of 8 μg/ml (Fig. 1). However, some synonymous mutations were observed in both resistant and susceptible isolates, including Gly31Gly(GGC→GGG), and Asp93Asp(GAT→GAC).
among *M. intracellulare* isolates, and Gly16Gly(GGC→GGT), Ala36Ala(GCG→GCC), and Leu134Leu(CTG→TTG) among *M. avium* isolates.

**Cross-resistance between Cfz and bedaquiline.** The bedaquiline susceptibility outcomes and the cross-resistance profile between Cfz and bedaquiline are shown in Table 3. The bedaquiline resistance rates of *M. intracellulare*, *M. avium*, *M. kansasii*, *M. gordonae*, *M. abscessus*, and *M. fortuitum* were 11.4% (4/35), 0% (0/22), 2.2% (1/45), 18.2% (2/11), 10% (4/40), and 6.1% (2/33), respectively. Except for 2 *M. intracellulare* isolates, all the bedaquiline-resistant NTM isolates were resistant to Cfz as well. Conversely, almost all of the Cfz-resistant NTM isolates were still sensitive to bedaquiline. No nonsynonymous mutation in the *atpE* gene was detected among all the bedaquiline-resistant NTM isolates tested. One of the two Cfz-bedaquiline cross-resistant *M. intracellulare* isolates possessed an Ala153Pro mutation in the Rv0678 homologue.

### Table 1 MICs of CFZ against reference strains of 30 RGM species and 17 SGM species

| Strain by type | Mycobacterium species (strain) | MIC (µg/ml) |
|---------------|------------------------------|-------------|
| **RGM species** |                              |             |
| ATCC 19977    | *M. abscessus*                | 0.5         |
| ATCC 27406    | *M. agri*                     | 2           |
| ATCC 27280    | *M. aichiense*                | 0.125       |
| ATCC 23366    | *M. aurum*                    | 0.125       |
| ATCC 33464    | *M. australisficanum*          | 0.25        |
| ATCC 14472    | *M. chelone*                  | 0.5         |
| ATCC 19627    | *M. chitae*                   | 0.125       |
| ATCC 27278    | *M. chubuense*                | 0.25        |
| DSM 44829     | *M. cosmeticum*               | 4           |
| ATCC 19340    | *M. diemhoferi*               | 4           |
| ATCC 35219    | *M. fallax*                   | <0.0313     |
| ATCC 6841     | *M. fortuitum*                | 2           |
| ATCC 27726    | *M. gadium*                   | 0.0625      |
| ATCC 43909    | *M. gilvum*                   | 0.125       |
| ATCC BAA-955  | *M. goodii*                   | 2           |
| DSM 44124     | *M. mucogenicum*              | <0.0313     |
| ATCC 25795    | *M. neoaureum*                | 0.125       |
| ATCC 27023    | *M. obuense*                  | <0.0313     |
| ATCC 19686    | *M. parafortuitum*            | 1           |
| DSM 43271     | *M. peregrinum*               | 1           |
| ATCC 11758    | *M. phlei*                    | 2           |
| ATCC 33776    | *M. porcinum*                 | 2           |
| ATCC 35154    | *M. puloveris*                | 0.25        |
| ATCC 35796    | *M. senegalense*              | 0.25        |
| ATCC 700731   | *M. septicum*                 | 0.25        |
| ATCC 19420    | *M. smegmatis*                | 4           |
| ATCC 33027    | *M. sphagni*                  | 2           |
| ATCC 19527    | *M. thermoresistible*         | 1           |
| ATCC 27282    | *M. tokaiense*                | 0.25        |
| ATCC 15483    | *M. vaccae*                   | 2           |
| **SGM species** |                              |             |
| ATCC 25276    | *M. asiaticum*                | 0.125       |
| ATCC 25291    | *M. avium*                    | <0.0313     |
| DSM 44243     | *M. celatum*                  | <0.0313     |
| DSM 44622     | *M. chimaera*                 | 0.125       |
| ATCC 15754    | *M. gastr*                    | <0.0313     |
| ATCC 14470    | *M. gordonae*                 | <0.0313     |
| ATCC 13950    | *M. intracellulare*           | <0.0313     |
| ATCC 12478    | *M. kansasi*                  | <0.0313     |
| ATCC 19422    | *M. microti*                  | <0.0313     |
| ATCC 19530    | *M. nonchromogenicum*         | 0.0625      |
| DSM 44648     | *M. parascrofulaceum*         | <0.0313     |
| ATCC 19981    | *M. scrofulaceum*             | <0.0313     |
| ATCC 35799    | *M. szulgai*                  | <0.0313     |
| ATCC 15755    | *M. terrae*                   | 0.25        |
| ATCC 23292    | *M. triviale*                 | >8          |
| ATCC 27294    | *M. tuberculosis* (H37Rv)     | 0.25        |
| ATCC 19250    | *M. xenopi*                   | <0.0313     |

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DISCUSSION

NTM can cause diseases of respiratory tract, skin, soft tissue, and so on. The incidence of NTM disease has been continuously growing worldwide in recent years (14, 15). Unfortunately, most of the NTM species demonstrate strong resistance to many antimicrobial agents (16). More candidate drugs are need to establish potent regiments for NTM treatment. Cfz was used to replace rifampin in a study to treat lung disease caused by \textit{M. avium}, and equivalent efficacy was observed (10). For the treatment of infection caused by \textit{M. abscessus}, Yang et al. showed that only 24% (10/42) of the

![Figure 1](attachment:figure1.png)

FIG 1 (a to f) MIC distributions of the six most prevalent NTM species. Strains in panels a and b were stratified based on the sequences of the MarR homologues.

| Category | Species | No. of isolates | MIC(s) (no. of isolates) (µg/ml)|
|----------|---------|-----------------|---------------------------------|
| SGM      | \textit{M. arupense} | 4               | <0.0313, 2, 0.0625, >8          |
|          | \textit{M. xenopi}    | 4               | <0.0313, 0.0016, 0.5, 1         |
|          | \textit{M. szulgai}   | 3               | <0.0313 (2), 0.0625             |
|          | \textit{M. fuerth}    | 2               | 0.5, 8                          |
|          | \textit{M. parascrofulaceum} | 1         | 2                               |
|          | \textit{M. terrae}    | 1               | <0.0313                         |
|          | \textit{M. malmoense} | 1               | <0.0313                         |
| RGM      | \textit{M. chelonae}  | 3               | 0.25, 0.5, 8                    |
|          | \textit{M. holsaticum} | 1            | <0.0313                         |
|          | \textit{M. massiliense} | 3             | 2 (2), >8                       |

*Numbers of isolates are 1 unless stated otherwise in parentheses.*
patients achieved conversion of culture-negative sputum with Cfz-containing regimen, demonstrating the weak potency of Cfz against _M. abscessus_ infection (17). In this study, we first evaluated the efficacy of Cfz against clinical NTM isolates collected from mainland China. Although Cfz has been used for the treatment of MDR-TB and NTM diseases, there is no agreement on the breakpoint of Cfz susceptibility testing for _M. tuberculosis_, nor for NTM. Defining the ECOFF value based on data produced by multiple centers with a large sum of strains is an important step for establishing breakpoints, whereas the MIC data of different NTM species against Cfz are very scarce right now.

In this study, SGM and RGM demonstrated different susceptibility profiles against Cfz in vitro. Cfz presented excellent inhibitory activity against the overwhelming majority of the recruited reference strains of SGM; 16 out of the 17 species tested had MICs of ≤0.25 μg/ml, and 11 of the MICs were below 0.1 μg/ml. However, the MICs of different RGM species were very different. Seventeen out of the 30 tested reference strains of RGM species had MICs lower than 1 μg/ml, and four of them were even below 0.1 μg/ml, whereas three strains had MICs at 4 μg/ml, and the other 10 MICs were in

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**FIG 2** Sequence alignment outcome of the Rv0678 homologue proteins. Alignments of the amino acid sequences of _M. tuberculosis_, _M. avium_, _M. kansasii_, _M. fortuitum_, _M. intracellulare_, and _M. abscessus_ are shown. The topology of the Rv0678-encoded protein of _M. tuberculosis_ is shown at the top. Red boxes with white letters indicate a single, fully conserved residue. Blue frames indicate highly conserved residues. β-Strands are rendered as arrows.
the middle. According to the MICs of different reference strains, Cfz has inhibitory effects on the growth of most RGM species but with less stronger potency than with SGM. Our testing with clinical isolates produced consistent outcomes with the reference strains. Cfz exhibited very good inhibitory activity against most of the tested clinical SGM isolates, especially the most prevalent very pathogenic species, like *M. kansasii* and *M. avium* complex (MAC). However, the most isolated RGM species, including *M. abscessus* and *M. fortuitum* isolates, had high MIC values for Cfz. The susceptibility results of RGM against Cfz in the limited publications from other authors were very conflicting. Shen et al. (11) reported that Cfz had very strong efficacy against RGM, with an MIC90 value at 0.5 \( \mu g/ml \) for *M. abscessus*, and 99.1% of the recruited strains had MICs of \( \leq 1 \mu g/ml \) in vitro. Furthermore, Cfz had efficacy against *M. fortuitum* and *M. chelonae* that was comparable to that of *M. abscessus* (11). Li et al. (13) adopted the cytotoxic concentration (CC) of \( \leq 1 \mu g/ml \) from Shen et al. (11), but very paradoxical outcomes were obtained. All the reference strains of different RGM species had extremely low MICs, but the clinical isolates of *M. abscessus*, *M. massiliense*, and *M. fortuitum* had resistance rates of 26.4%, 55.6%, and 22.2%, respectively. The MIC90 values for those three RGM species were 8 \( \mu g/ml \), 16 \( \mu g/ml \), and 256 \( \mu g/ml \), respectively (13). However, van Ingen et al. found that Cfz resistance among isolates of *M. abscessus* and *M. fortuitum* were quite common, with resistance rates of 90% (74/82) and 46% (21/46) when applying the breakpoint at 2 \( \mu g/ml \), respectively (8). Our results were in accordance with the assay of van Ingen (8). Although a small proportion of the clinical isolates of *M. abscessus* and *M. fortuitum* had MICs of \( \leq 0.5 \mu g/ml \), the majority of the isolates did not respond well against Cfz in vitro. Therefore, we did not assess the ECOFFs for them. The plausible reason for those paradoxical outcomes within same species is elusive. More studies from different teams and different regions are urgently needed to justify the actual reason.

Due to the lack of a well-recognized method and breakpoint for Cfz susceptibility testing for *M. tuberculosis* and NTM, the breakpoints adopted in different studies were different (12, 13). Cowman et al. adopted a uniform 0.8 \( \mu g/ml \) breakpoint for different SGM species using the Bactec 460 system, and Cfz showed good activity against most SGM species (12). Among them, the susceptibility rates against Cfz were 74% and 88% for MAC and *M. kansasii*, respectively. van Ingen et al. acquired higher susceptibility rates in their assay, at 92% and 100% for MAC and *M. kansasii*, respectively, using a

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**TABLE 3** Bedaquiline susceptibility testing outcomes and cross-resistance profiles between CFZ and bedaquiline against six most prevalent NTM species

| Species          | BDQ resistance | No. of isolates against CFZ that were:<br>Resistant | Sensitive |
|------------------|----------------|-----------------------------------------------|-----------|
| *M. avium*       | R              | 0                                             | 0         |
|                  | S              | 3                                             | 19        |
| *M. intracellulare* | R            | 2                                             | 2         |
|                  | S              | 11                                            | 20        |
| *M. kansasii*    | R              | 1                                             | 0         |
|                  | S              | 0                                             | 44        |
| *M. gordonae*    | R              | 2                                             | 0         |
|                  | S              | 0                                             | 9         |
| *M. abscessus*   | R              | 4                                             | 0         |
|                  | S              | 26                                            | 10        |
| *M. fortuitum*   | R              | 2                                             | 0         |
|                  | S              | 25                                            | 6         |

\(^a\)The breakpoints for bedaquiline were 1.0 \( \mu g/ml \) and 2.0 \( \mu g/ml \) for SGM and RGM, respectively. R, resistant; S, sensitive.

\(^b\)The breakpoint for CFZ was 1.0 \( \mu g/ml \).
Middlebrook 7H10 agar dilution method with breakpoint at 2 μg/ml (6). The tentative ECOFFs defined in our assay for *M. kansasii*, *M. avium*, and *M. intracellularare* were 0.5 μg/ml, 1 μg/ml, and 2 μg/ml, respectively. To our knowledge, this is the first report on defining ECOFFs for NTM species for Cfz; thus, more validations are need. A study from Diacon et al. showed that the maximum concentration of drug in serum \(C_{\text{max}}\) of Cfz was around 0.25 μg/ml when administered alone or together with other drugs (18), whereas another study conducted among healthy adults obtained a serum concentration of about 0.8 μg/ml (19). The overall pharmacokinetic data are still too limited, but the available data demonstrated that although Cfz could theoretically inhibit the growth of most SGM NTM species and some RGM species in vivo, the frequently used 1 to 2 μg/ml CC by different studies might not be justified. More studies are urgently needed to address this important issue.

The Rv0678-encoded protein is a transcriptional repressor for MmpL transport systems (6). Mutations in Rv0678 can upregulate the multisubstrate efflux pump MmpL5, which leads to Cfz resistance (5). Zhang et al. showed that 97% (93/96) of Cfz-resistant *M. tuberculosis* strains had mutations in Rv0678 (20), whereas we did not find a specific mutation that obviously was related to Cfz resistance in the 3 Cfz-resistant *M. avium* isolates. Among the 13 Cfz-resistant *M. intracellularare* isolates, two isolates (15.4%) with MICs of ≥8 μg/ml possessed Asp92Glu and Ala153Pro mutations in their Rv0678 homologues, respectively. Multiple-amino-acid alignment showed that the locus Asp92 was conserved among different NTM species (Fig. 2). According to the crystal structure of the Rv0678 protein of *M. tuberculosis* (PDB 4NB5), Asp92 located in a DNA-protein binding domain, which is the active site of the MarR family. Ala153 located in loop α6, which was involved in dimerization of the Rv0678-encoded protein, so an Ala153Pro mutation would increase the steric hindrance leading to decreased activity of the protein (6). Bioinformatics analysis suggested that mutations in the conserved amino acid locus were always associated with drug resistance, which was in line with our results. On the other hand, mutations located in the nonconserved domains seemed not to associate with Cfz resistance. Asp127Asn in *M. avium* and Ala138Pro in *M. intracellularare* were found in both Cfz-susceptible and -resistant isolates, and both mutations located in the flexible region of the η1 turn, which was more tolerant of single-amino-acid mutations. Generally, efflux pump mutations are related to low-level drug resistance, while high-level drug resistance is attributed to mutations in the target gene. In our study, although 2 out of 9 high-level Cfz-resistant isolates (MIC, ≥8 μg/ml) of *M. intracellularare* had a Rv0678 homologous gene mutations, no such mutation was observed in *M. avium*. Hence, MarR homologues might not be the only target for Cfz to explore its bacteriostatic activity.

Previous studies reported that *M. tuberculosis* strains had cross-resistance between Cfz- and bedaquiline-resistant strains selected in vitro, and Rv0678 mutation was associated with cross-resistance (5, 21). For NTM, these phenomena remain to be determined. In this assay, single-direction cross-resistance between Cfz- and bedaquiline-resistant isolates was observed. The Cfz-resistant isolates of different NTM species were still sensitive to bedaquiline, except for two *M. intracellularare* isolates. On the other hand, all bedaquiline-resistant isolates were also resistant to Cfz in vitro (Table 3). An atpE mutation was not found among all the bedaquiline-resistant NTM isolates, and this is consistent with a previous study showing that no atpE mutation existed among NTM strains isolated from patients (22). The Ala153Pro mutation in the Rv0678 homologue was detected in one *M. intracellularare* isolate with Cfz and bedaquiline cross-resistance. Although little is known about the cross-resistance status among NTM, these findings may have some clinical implications and warrant further evaluation of clinical isolates with reduced susceptibilities to each drug. PepQ, a putative Xaa-Pro aminopeptidase, has also been identified as conferring cross-resistance between bedaquiline- and Cfz-resistant strains of *M. tuberculosis* (5). Due to the unsuccessful amplification of the pepQ gene, we could not include this gene in our study.

In conclusion, our data demonstrate that Cfz had high in vitro activity against both reference strains and clinical isolates of many SGM species. Although clinical isolates of
M. abscessus and M. fortuitum had very high resistance rates against Cfz, 17 out of the 30 reference strains of RGM species we tested also had MICs below 1 μg/ml in vitro. According to the tentative ECOFF data in our assay and pharmacokinetic data from other reports, 1 to 2 μg/ml could be temporarily proposed as the breakpoint for NTM susceptibility testing for Cfz. In addition, mutations in the Rv0678 homologous genes of M. intracellulare may somewhat confer Cfz resistance. Moreover, single-direction cross-resistance between bedaquiline and Cfz was observed among the tested NTM isolates. Our study provided important insights on the clinical application of Cfz for the treatment of NTM infections.

MATERIALS AND METHODS

Reference strains and clinical isolates. All the mycobacterial reference strains stored in the Bio-bank in Beijing Chest Hospital (Beijing, China) were tested against Cfz in vitro, including 30 rapidly growing mycobacterium (RGM) species and 17 slowly growing mycobacterium (SGM) species. These reference strains were obtained either from the American Type Culture Collection (ATCC) or from the German Collection of Microorganisms (DSM). The species constitution of these reference strains is listed in Table 1. A total of 209 NTM clinical isolates, collected between 2015 and 2016, were recruited, including 40 M. abscessus isolates, 35 M. intracellulare isolates, 33 M. fortuitum isolates, 22 M. avium isolates, 45 M. kansasi isolates, and 11 M. gordonae isolates. The species constitution of the remaining 23 isolates is presented in Table 2. The isolates were differentiated to the species level by both growth test on medium containing p-nitrobenzoic acid and by 16S rRNA gene, hsp65, rpoB, and 16-23S rRNA internal transcribed spacer (ITS) sequencing (22).

MIC testing. Cfz and bedaquiline were purchased from Lyhe-Pharmaceutical (Nanjing, China) and Shanghai Biochempartner Co., Ltd. (Shanghai, China), respectively. Both drugs were dissolved in dimethyl sulfoxide (DMSO). A broth microdilution method was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (23). 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 inoculum was prepared with fresh culture grown on Lowenstein-Jensen (L-J) medium. The broth microdilution format was set up as a 2-fold dilution, and the concentration range for Cfz was 0.0313 μg/ml to 8 μg/ml, while that for bedaquiline was 0.0078 μg/ml to 2 μg/ml. According to Pang’s study, the critical concentrations for bedaquiline susceptibility testing were 1.0 μg/ml and 2.0 μg/ml for SGM and RGM, respectively (22). In order to assess the cross-resistance between bedaquiline and Cfz, a tentative critical concentration at 1.0 μg/ml was applied for Cfz regardless of the NTM species. Briefly, a bacterial suspension of 0.5 McFarland standard was prepared and then diluted and inoculated into a 96-well microtiter plate to achieve final bacterial load of 10^6 CFU per well. Plates were then incubated at 37°C for 7 days for SGM and 3 days for RGM. A 70-μl solution containing 20 μl alamarBlue and 50 μl Tween 80 (5%) was added to each well and incubated for 24 h at 37°C before assessing color development. A change from blue to pink or purple indicated bacterial growth (24). The MIC was defined as the lowest concentration of antibiotic that prevented a color change from blue to pink.

ECOFF determination. For species with enough isolates and for which Cfz demonstrated good inhibitory activity, the ECOFF was determined according to the distribution profile of the MIC values. For a unimodal MIC distribution profile, in which MICs presented as a single-peak pattern, ECOFF was defined as concentration that could inhibit >95% of the bacterial population; for a bimodal MIC distribution profile, in which MICs presented as a double-peak pattern, the ECOFF was set between the two populations.

Alignment of MarR homologues of different NTM species. Rv0678 of M. tuberculosis encodes a protein homologous to MarR of Escherichia coli. Mutations in Rv0678, causing overexpression of the efflux pump, resulted in Cfz resistance (20). The sequences of Rv0678 homologous genes of M. avium, M. kansasi, M. fortuitum, M. intracellulare, and M. abscessus were obtained from the NCBI database. Multiple-sequence alignment of the homologous proteins was performed using the Clustal Omega software. Structure-based multiple-sequence alignment was performed with ESPript 3 (http://espript.ibcp.fr/ESPr ipt/ESPript/) based on the crystal structure of the Rv0678 protein of M. tuberculosis.

DNA amplification and sequencing. The atpE gene encodes the ATP synthase subunit to which bedaquiline binds, and bedaquiline-resistant strains selected in vitro often have mutations in atpE. Sequence analysis of the atpE gene of bedaquiline-resistant isolates of the six most prevalent NTM species in this assay and Rv0678 homologous genes of all the M. avium and M. intracellulare isolates was performed. The target genes of the corresponding reference strains of the involved species were also sequenced, and a mutation was defined as a difference from the sequences of the reference strains. The primers used for PCR amplification are listed in Table S1 in the supplemental material. The amplification products were sequenced by the Tsingke Company (Beijing, China).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00072-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.
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We declare no conflicts of interest.

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