



# *In Vitro* Activities of Bedaquiline and Delamanid against Nontuberculous Mycobacteria Isolated in Beijing, China

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**ABSTRACT** Due to the natural resistance of nontuberculous mycobacteria (NTM) against multiple antibiotics, treatment of infections caused by them is often long-course and less successful. The main objective of our study was the evaluation of *in vitro* susceptibility of 209 isolates consisting of different NTM species against bedaquiline and delamanid. Furthermore, reference strains of 33 rapidly growing mycobacterium (RGM) species and 19 slowly growing mycobacterium (SGM) species were also tested. Bedaquiline exhibited strong *in vitro* activity against both reference strains and clinical isolates of different SGM species, as the majority of the strains demonstrated MICs far below 1  $\mu\text{g/ml}$ . Bedaquiline (Bdq) also exhibited potent activity against the recruited RGM species. A total of 29 out of 33 reference RGM strains had MICs lower than 1  $\mu\text{g/ml}$ . According to the MIC distributions, the tentative epidemiological cutoff (ECOFF) values, and the pharmacokinetic data, a uniform breakpoint of 2  $\mu\text{g/ml}$  was temporarily proposed for NTM's Bdq susceptibility testing. Although delamanid (Dlm) was not active against most of the tested reference strains and clinical isolates of RGM species, it exhibited highly variable antimicrobial activities against the 19 tested SGM species. Eleven species had MICs lower than 0.25  $\mu\text{g/ml}$ , and 7 species had MICs greater than 32  $\mu\text{g/ml}$ . Large numbers of *M. kansasii* (39/45) and *M. goodii* (6/10) clinical isolates had MICs of  $\leq 0.125$   $\mu\text{g/ml}$ . This study demonstrated that bedaquiline had potent activity against different NTM species *in vitro*, and delamanid had moderate activity against certain species of SGM. The data provided important insights on the possible clinical application of Bdq and Dlm to treat NTM infections.

**KEYWORDS** bedaquiline, delamanid, nontuberculous mycobacteria, susceptibility

Nontuberculous mycobacteria (NTM) are a group of organisms within the genus *Mycobacterium*, except for the *Mycobacter tuberculosis* complex and *Mycobacter leprae*, which can cause tuberculosis-like diseases in the lung or other organs (1). During the past decades, the prevalence of lung infections caused by NTM has increased globally and has even surpassed tuberculosis (TB) in some countries; thus, it emerged as a new threat to human health (2–5). Regimens targeting the most prevalent NTM species have been recommended by some professional institutions but are usually weakly evidence based. Therefore, the treatment outcomes of NTM infections are often unsatisfactory with high chances of failure, relapse, and default (6–8). In addition, treatment options after failure of the first-line drug regimens are scarce, and the treatment with alternative drugs combinations are yet to be established. Hence, the continuous screening of new anti-NTM medications is necessary.

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Bedaquiline (Bdq) and delamanid (Dlm) are two new anti-TB drugs that have recently been demonstrated to have strong efficacies against multiple drug-resistant tuberculosis (MDR-TB) members, both *in vitro* and *in vivo*. Based on the genotypic and phenotypic similarities within the genus *Mycobacterium*, those two drugs might also have bacterial inhibitory activities against different NTM species, although only limited data are available to justify this deduction. Bdq is an oral antimycobacterial agent belonging to a new class of drugs called diarylquinolines. It has low equivalent MICs for some NTM species (2, 9, 10), and a pilot study using a Bdq-containing regimen to treat a few patients infected with *Mycobacterium avium* complex (MAC) or *Mycobacterium abscessus* favored the potential clinical and microbiological activity of Bdq (11). Dlm, a new agent derived from the nitro-dihydro-imidazooxazole class of compounds that inhibits mycolic acid synthesis, has strong *in vitro* activity against *M. tuberculosis*, including MDR-TB strains (12–14). Limited studies have demonstrated that Dlm harbors strong inhibitory activities against clinical isolates of MAC (15). Understanding the activities of Dlm against different NTM species also remains limited.

To better understand the efficacy of Bdq and Dlm against different NTM species, we determined the MICs of 52 NTM reference strains and 209 NTM isolates collected in Beijing, China. In addition, we attempted to define the tentative epidemiological cutoff (ECOFF) values for the most prevalent NTM species, which is an important factor in setting up the breakpoint for a drug susceptibility testing. Furthermore, we investigated the potential molecular mechanism of Dlm resistance among different NTM species. The aim of our study was to facilitate drug selection for treating NTM infections, especially for the species with high prevalence.

## RESULTS

**MICs and ECOFFs of Bdq against NTM strains.** The MICs of the 52 reference strains against Bdq are presented in Table 1. Bdq demonstrated uniform strong antibacterial activity against almost all of the tested SGM species, with MICs far below 1  $\mu\text{g/ml}$ , except *Mycobacterium rhodesiae* (MIC, 1  $\mu\text{g/ml}$ ), *Mycobacterium xenopi* (MIC, 2  $\mu\text{g/ml}$ ), and *Mycobacterium celatum* (MIC, >32  $\mu\text{g/ml}$ ). Furthermore, Bdq also exhibited very potent *in vitro* activity against the recruited RGM reference strains; 29 out of 33 RGM species had MICs of  $\leq 1$   $\mu\text{g/ml}$ , and 18 of them had MICs of  $\leq 0.25$   $\mu\text{g/ml}$ . Among the four leftover RGM species, one had MICs of 2  $\mu\text{g/ml}$ , and the other three had MICs of >2  $\mu\text{g/ml}$ .

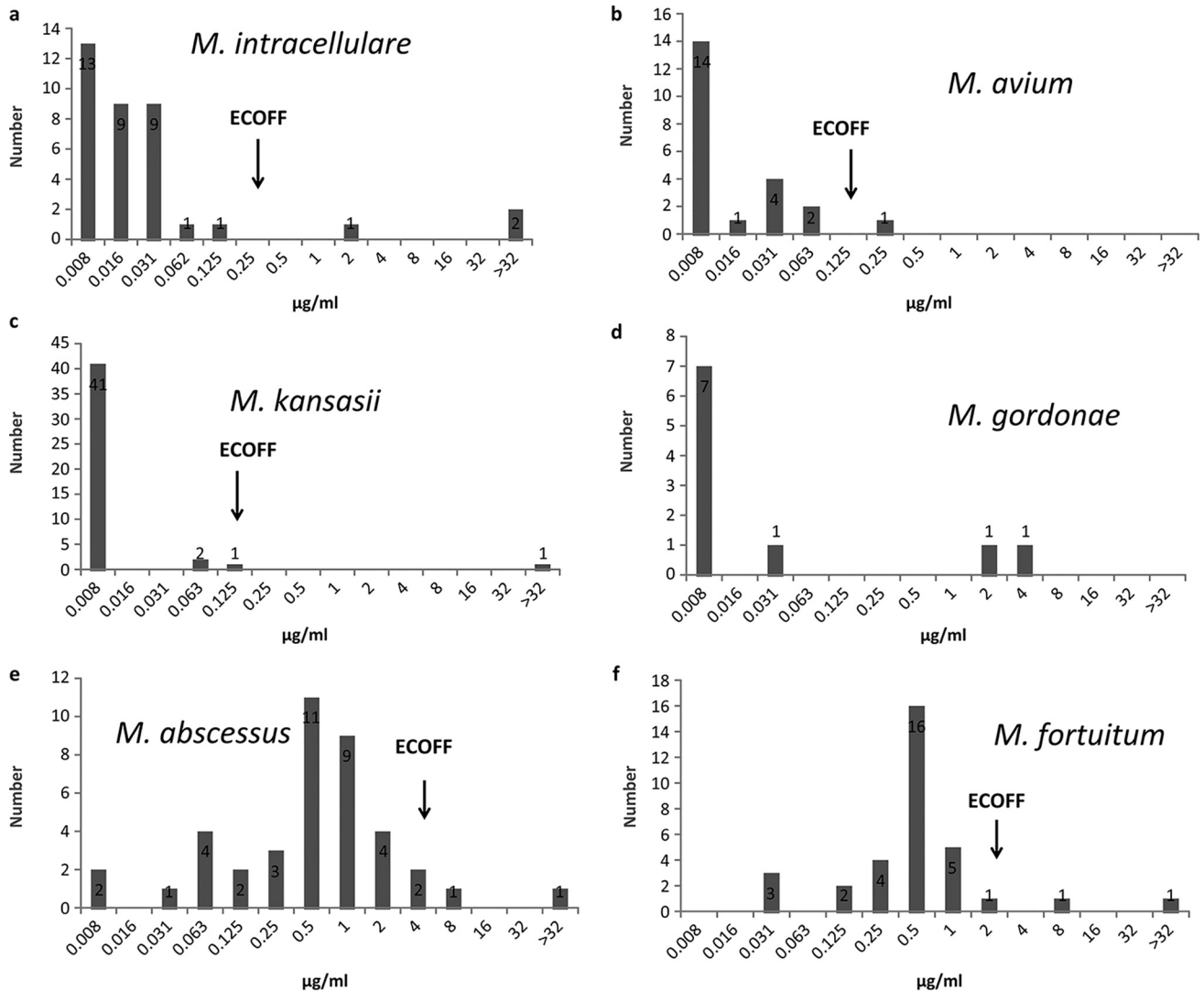
The MIC distributions of the most prevalent NTM species against Bdq are shown in Fig. 1. The susceptibility profile of the clinical isolates to Bdq was in concordance with that of the reference strains, i.e., strong antibacterial activity against the absolute majority isolates of SGM for all the included species. Similar activities were demonstrated against the included RGM isolates of different species but with relatively higher MIC values in contrast with SGM. Among the recruited SGM species, Bdq exhibited the strongest activity against *Mycobacterium kansasii*, with a tentative ECOFF at 0.125  $\mu\text{g/ml}$ , whereas the overwhelming majority of the isolates had MICs below 0.008  $\mu\text{g/ml}$ . Bdq also demonstrated strong activities against *M. avium* and *M. intracellulare*, with tentative ECOFFs at 0.125  $\mu\text{g/ml}$  and 0.25  $\mu\text{g/ml}$ , respectively. The activity of Bdq against *Mycobacterium gordonae* was discrete. Eight isolates had MICs lower than 0.031  $\mu\text{g/ml}$ , while another two had MICs of  $\geq 2$   $\mu\text{g/ml}$ . Due to the limited number of isolates, the ECOFF of *M. gordonae* was not determined. Among the recruited RGM species, Bdq harbored good activities against *M. abscessus* and *Mycobacterium fortuitum*, with MIC<sub>90</sub> values of 2  $\mu\text{g/ml}$  and of 1  $\mu\text{g/ml}$ , respectively. The ECOFF for *M. abscessus* and *M. fortuitum* were at 4  $\mu\text{g/ml}$  and 2  $\mu\text{g/ml}$ , respectively. The susceptibility testing outcomes for species with less than five isolates are shown in Table 2. Although there were small numbers for each species, Bdq presented the same inhibitory tendency against them.

**MICs and ECOFF of Dlm against NTM strains.** Dlm exhibited highly variable antimicrobial activities against the 19 tested SGM species. Although, 11 species had MICs lower than 0.25  $\mu\text{g/ml}$ , 7 species had MICs greater than 32  $\mu\text{g/ml}$ , and 1 species

**TABLE 1** MICs of Bdq and Dlm against reference strains of 33 RGM species and 19 SGM species

Strain by <i>Mycobacterium</i> type	Species (strain)	MIC ( $\mu\text{g/ml}$ ) by antimicrobial agent	
		Bdq	Dlm
<b>RGM</b>			
ATCC 19977	<i>Mycobacterium abscessus</i>	0.5	>32
ATCC 27406	<i>Mycobacterium agri</i>	0.016	1
ATCC 27280	<i>Mycobacterium aichiense</i>	0.031	8
ATCC 23366	<i>Mycobacterium aurum</i>	0.25	>32
ATCC 33464	<i>Mycobacterium austroafricanum</i>	0.125	>32
ATCC 14472	<i>Mycobacterium chelonae</i>	2	>32
ATCC 19627	<i>Mycobacterium chitae</i>	1	>32
ATCC 27278	<i>Mycobacterium chubuense</i>	0.5	>32
DSM 44829	<i>Mycobacterium cosmeticum</i>	1	>32
ATCC 19340	<i>Mycobacterium diernhoferi</i>	<0.008	>32
ATCC 35219	<i>Mycobacterium fallax</i>	<0.008	>32
ATCC 35753	<i>Mycobacterium farcinogenes</i>	<0.008	>32
ATCC 14474	<i>Mycobacterium flavescens</i>	>32	>32
ATCC 6841	<i>Mycobacterium fortuitum</i>	0.5	>32
ATCC 27726	<i>Mycobacterium gadium</i>	1	<0.125
ATCC 43909	<i>Mycobacterium gilvum</i>	0.016	>32
ATCC BAA-955	<i>Mycobacterium goodii</i>	1	>32
DSM 44124	<i>Mycobacterium mucogenicum</i>	0.25	>32
ATCC 25795	<i>Mycobacterium neoaurum</i>	0.5	>32
ATCC 27023	<i>Mycobacterium obuense</i>	<0.008	1
ATCC 19686	<i>Mycobacterium parafortuitum</i>	1	>32
DSM 43271	<i>Mycobacterium peregrinum</i>	0.125	>32
ATCC 11758	<i>Mycobacterium phlei</i>	0.125	>32
ATCC 33776	<i>Mycobacterium porcinum</i>	0.5	>32
ATCC 35154	<i>Mycobacterium pulveris</i>	16	<0.125
ATCC 35796	<i>Mycobacterium senegalense</i>	0.25	>32
ATCC 700731	<i>Mycobacterium septicum</i>	0.016	>32
ATCC 25275	<i>Mycobacterium simiae</i>	0.016	>32
ATCC 33027	<i>Mycobacterium sphagni</i>	0.5	>32
ATCC 19420	<i>Mycobacterium smegmatis</i>	0.25	>32
ATCC 19527	<i>Mycobacterium thermoresistibile</i>	4	>32
ATCC 27282	<i>Mycobacterium tokaiense</i>	0.125	>32
ATCC 15483	<i>Mycobacterium vaccae</i>	0.25	>32
<b>SGM</b>			
ATCC 25276	<i>Mycobacterium asiaticum</i>	0.031	>32
ATCC 25291	<i>Mycobacterium avium</i>	<0.008	0.25
DSM 44243	<i>Mycobacterium celatum</i>	>32	2
DSM 44622	<i>Mycobacterium chimaera</i>	<0.008	<0.125
ATCC 15754	<i>Mycobacterium gastri</i>	<0.008	0.25
ATCC 14470	<i>Mycobacterium gordonae</i>	<0.008	<0.125
ATCC 13950	<i>Mycobacterium intracellulare</i>	<0.008	>32
ATCC 12478	<i>Mycobacterium kansasii</i>	<0.008	<0.125
ATCC 29571	<i>Mycobacterium malmoense</i>	0.031	<0.125
ATCC 19422	<i>Mycobacterium microti</i>	<0.008	<0.125
ATCC 19530	<i>Mycobacterium nonchromogenicum</i>	0.016	>32
DSM 44648	<i>Mycobacterium parascrofulaceum</i>	0.031	0.25
ATCC 27024	<i>Mycobacterium rhodesiae</i>	1	>32
ATCC 19981	<i>Mycobacterium scrofulaceum</i>	0.016	>32
ATCC 35799	<i>Mycobacterium szulgai</i>	0.016	<0.125
ATCC 15755	<i>Mycobacterium terrae</i>	<0.008	>32
ATCC 23292	<i>Mycobacterium triviale</i>	0.5	>32
ATCC 27294	<i>Mycobacterium tuberculosis</i> (H37Rv)	0.031	<0.125
ATCC 19250	<i>Mycobacterium xenopi</i>	2	<0.125

had an MIC of 2  $\mu\text{g/ml}$ . However, Dlm did not present evident inhibitory activity against most of the tested RGM species; 28 out of 33 tested RGM isolates had MICs greater than 32  $\mu\text{g/ml}$ . Only four RGM species had MICs below 2  $\mu\text{g/ml}$ , i.e., *Mycobacterium gadium* and *Mycobacterium pulveris* (MIC, <0.125  $\mu\text{g/ml}$ ) and *Mycobacterium agri* and *Mycobacterium obuense* (MIC, 1  $\mu\text{g/ml}$ ).



**FIG 1** The MIC distributions of Bdq against the six most prevalent NTM species.

The MIC distributions of Dlm against the most prevalent NTM species are shown in Fig. 2. The susceptibility profiles of clinical isolates and reference strains against Dlm showed concordance, i.e., partial antibacterial activity against SGM, but little activity against RGM isolates. Among the six most frequently isolated SGM species, Dlm

**TABLE 2** The MICs of Bdq and Dlm against clinically isolated species with less than 5 isolates

Category	Species	No. of isolates	MIC(s) (µg/ml) by antimicrobial agent (no. of isolates) <sup>a</sup>	
			Bdq	Dlm
SGM	<i>Mycobacterium arupense</i>	4	<0.0078 (2), 0.0625, 4	0.5, >32 (3)
	<i>M. xenopi</i>	4	0.0313, 0.0016, 0.5, 1	<0.125 (3), >32
	<i>M. szulgai</i>	3	<0.0078 (3)	<0.125 (2), 0.5
	<i>Mycobacterium fuerth</i>	2	<0.0078, 2	>32 (2)
	<i>M. parascrofulaceum</i>	1	<0.0078	16
	<i>M. terrae</i>	1	<0.0078	>32
	<i>M. malmoense</i>	1	<0.0078	<0.125
RGM	<i>M. chelonae</i>	3	0.0313, 0.0625, >32	>32 (3)
	<i>Mycobacterium holsaticum</i>	1	1	<0.125
	<i>Mycobacterium massiliense</i>	3	0.25 (2), 1	>32 (3)

<sup>a</sup>No. of isolates is 1 unless stated otherwise in parentheses.

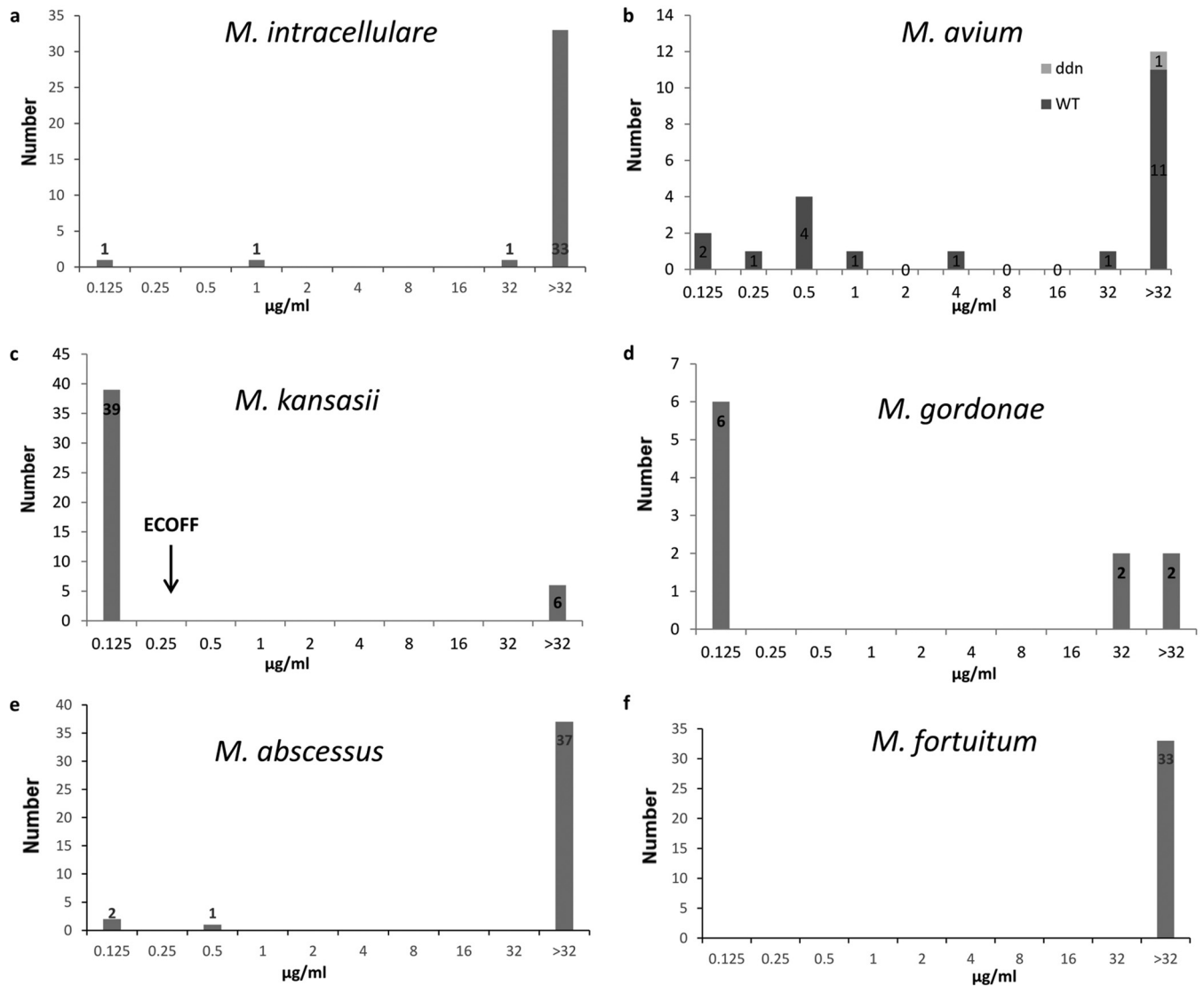
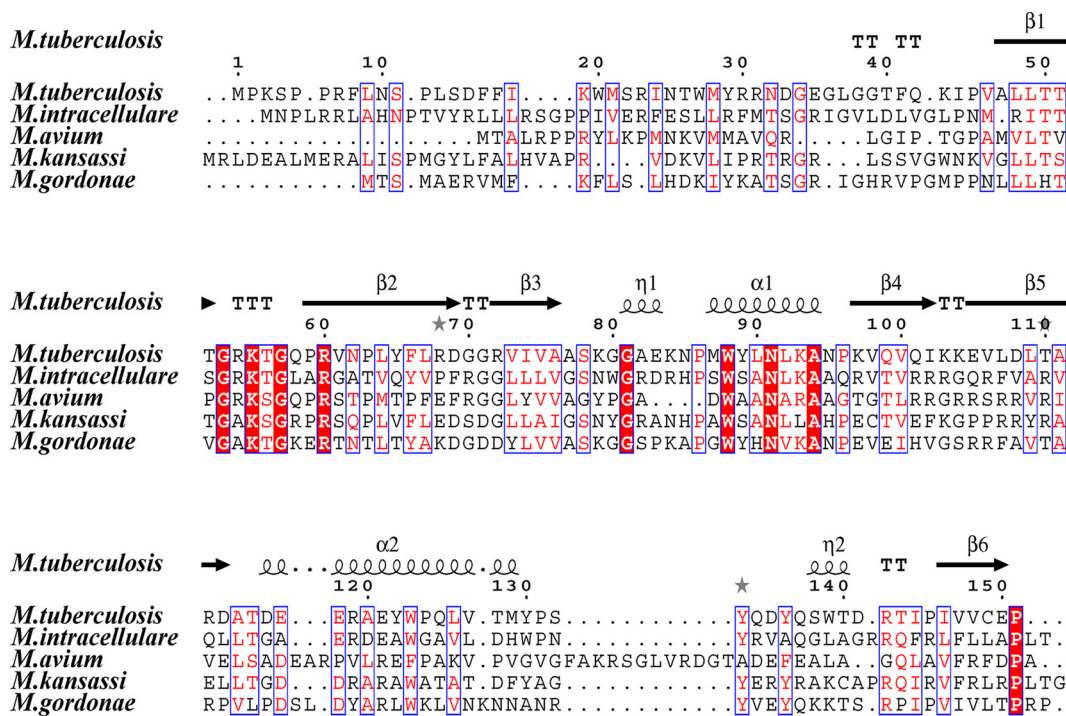


FIG 2 The MIC distributions of Dlm against the six most prevalent NTM species.

exhibited the strongest activity against *M. kansasii* with a tentative ECOFF at 0.25  $\mu\text{g/ml}$ . The activity of Dlm against *M. goodsonae* isolates was very different. While six isolates had MICs lower than 0.125  $\mu\text{g/ml}$ , another four had MICs of  $\geq 32$   $\mu\text{g/ml}$ . Among the 22 *M. avium* isolates, 7 had MICs of  $\leq 0.25$   $\mu\text{g/ml}$ , while the remaining 15 had MICs of  $\geq 0.5$   $\mu\text{g/ml}$ . Dlm did not present good inhibitory activity against *M. intracellulare* and *M. abscessus*, and the majority of the isolates were highly resistant to Dlm except a few isolates which had MICs of  $\leq 1$   $\mu\text{g/ml}$ . Furthermore, Dlm harbored the least inhibitory activities against *M. fortuitum*, as all of the 33 isolates had MICs greater than 32  $\mu\text{g/ml}$ . No ECOFF was determined for most of these species either due to a limited number of isolates or due to the weak activity of Dlm. In addition, the susceptibility testing outcomes for species with less than five isolates are presented in Table 2.

**Alignment and mutation of Ddn homologues.** Multiple amino acid alignment for the Ddn homologues of different mycobacterial species and the topology of proteins are shown in Fig. 3. The interactions between ddn and F420 included extensive hydrogen-bond and hydrophobic interactions, and most of the key amino acid involved are highly conserved among TB and NTM species, as indicated by the sequence alignment which includes Gly53, Lys55, Arg60, Trp88, and Asn91 (Fig. 3). In order to determine the relationship between mutations in *ddn* homologous genes and Dlm





**FIG 3** Sequence alignment outcome of the of Ddn homologue proteins. Alignment of the amino acid sequences of *M. tuberculosis*, *M. intracellulare*, *M. avium*, *M. kansasii*, and *M. gordonae*. The topology of the Rv3547 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.  $\beta$ -Strands are rendered as arrows.

resistance, the full-length *ddn* homologues of *M. avium* were sequenced. Among the 13 Dlm highly resistant *M. avium* isolates (MIC,  $\geq 32 \mu\text{g/ml}$ ), mutations within the Ddn homologues associated with Dlm resistance were identified, i.e., Thr79Arg and Ser86Cys which were detected in one *M. avium* isolate with MIC of  $>32 \mu\text{g/ml}$ . Furthermore, some synonymous mutations were also observed in drug resistance isolates, including Pro107Pro (CCC $\rightarrow$ CCT), Gly121Gly (GGG $\rightarrow$ GGA), Pro12Pro (CCG $\rightarrow$ CCC), and Arg73Arg (CGG $\rightarrow$ CGC). In addition, no mutation in *ddn* was detected in the remaining 9 isolates with MIC of  $\leq 4 \mu\text{g/ml}$ .

**DISCUSSION**

The prevalence of NTM infection has been noticed to be increasing, which may reflect the increasing awareness of physicians that leads to more frequent diagnosis or the improved laboratory capabilities (6). Most of the NTM species demonstrate strong resistance to multiple antimicrobial agents (16), and, as a consequence, treatment of NTM infections and establishment of an effective regimen are generally challenging. Identifying drugs potent against NTM infections is urgently needed. Bdq and Dlm are highly efficient treatments of drug-resistant tuberculosis; however, only limited data are available on their antibacterial activity against NTM species either *in vitro* or *in vivo*. In this study, we first evaluate the efficacy of Dlm against NTM isolates collected from mainland China. Furthermore, we also evaluated the activity of Bdq and Dlm against 52 reference strains of different mycobacterium species to gain insights on their potential use for specific NTM species. As they are new drugs, well-recognized susceptibility testing methods for Bdq and Dlm have not been developed, and breakpoints to define drug resistance for them have never been discussed prudently (9, 10). Defining the ECOFF value based on data produced by multiple centers with a large number of strains is an important step to establish the breakpoints. The MIC data of different NTM species against Bdq and Dlm still remain scarce.

In this study, Bdq presented excellent inhibitory activity against the overwhelming

majority of the recruited SGM reference strains and clinical isolates. A total of 16 out of 19 reference strains of SGM species exhibited MICs far below 1  $\mu\text{g/ml}$ . Bdq also exhibited very good inhibitory activities against most of the tested clinical SGM isolates, especially the most prevalent and highly pathogenic species, such as *M. kansasii* and *Mycobacterium avium* complex (MAC), as previously described (10). Pang et al. also demonstrated that Bdq had good inhibitory activities against clinical strains of some SGM species isolated from China (9). All the tested NTM isolates in this study were collected in four hospitals located in mideastern China from 2011 to 2015, while the NTM isolates in our study were collected in Beijing Chest Hospital located in northern China from 2015 to 2016. Despite the differences in isolate collection time, location, and sample size, Bdq exhibited uniform bactericidal activity against SGM in these two studies. Notably, Bdq also exhibited good activity against the recruited RGM. A total of 29 out of 33 RGM species had MICs lower than 1  $\mu\text{g/ml}$ , and most clinical isolates of different RGM species also had MICs lower than 1  $\mu\text{g/ml}$ , including the most drug-resistant species, such as *M. abscessus* and *M. fortuitum*. RGM infection treatment is often very difficult because the bacteria can have more severe drug resistance patterns than that of SGM bacteria, and drugs with strong potency *in vitro* have not been reported yet. Although only a few studies report the clinical use of Bdq on NTM infection, based on our results, an extrapolation of its usage to treat infection caused by different SGM species is quite reasonable. However, further clinical trials are required to identify its real efficacy for patient care.

In contrast with Bdq, Dlm showed less strong antibacterial activity against either RGM or SGM. Almost all of the involved RGM reference strains and clinical isolates had very high MICs against Dlm, suggesting a low chance to be active for RGM infection treatment. Considering SGM, Dlm presented very different inhibitory activity against different species. Eleven of the 19 tested reference strains of different SGM species had MICs below 0.25  $\mu\text{g/ml}$ , while 7 species had MICs of  $\geq 32$   $\mu\text{g/ml}$ . For clinical isolates, Dlm exhibited good inhibitory activities against the majority isolates of *M. kansasii* and *M. gordonae*, the minority of *M. avium*, but almost no inhibitory activity against *M. intracellulare*. The high MIC variance within or between different NTM species highlighted the need for susceptibility testing before Dlm administration. A recent study with 20 isolates of MAC showed that all the tested *M. intracellulare* isolates had MICs below 0.4  $\mu\text{g/ml}$ , which is not consistent with our results (15). Differences in the choice of susceptibility testing method might have caused this discordance, while different origins and the small number of isolates tested could also have incurred deviations. More studies from different teams and regions will help to evaluate the efficacies of Dlm objectively.

Due to the lack of a well-recognized method and breakpoint for Bdq susceptibility testing for *M. tuberculosis* and NTM, the breakpoint adopted in various studies was different (17, 18). Pang et al. adopted a 2- $\mu\text{g/ml}$  breakpoint using the Microplate alamarBlue assay for RGM species and 1  $\mu\text{g/ml}$  for SGM (18). Brown-Elliott et al. showed that all of the 103 tested MAC isolates had Bdq MICs of  $\leq 0.03$   $\mu\text{g/ml}$  by broth microdilution using Mueller-Hinton (MH) broth (10). The tentative ECOFFs defined in our assay for *M. kansasii*, *M. avium*, and *M. intracellulare* were all  $\leq 1$   $\mu\text{g/ml}$  in our study and were 4  $\mu\text{g/ml}$  for *M. abscessus* and 2  $\mu\text{g/ml}$  for *M. fortuitum*. A 2-h postdosage study verified that the drug was well absorbed, and the median  $C_{max}$  of Bdq in 37 healthy volunteers was 3.39  $\mu\text{g/ml}$  (19). According to these pharmacokinetic data, Bdq could theoretically inhibit the growth of most SGM NTM species and many RGM species *in vivo*, although clinical experience on Bdq for NTM patient care is rare. By combining the ECOFF and pharmacokinetic data, a uniform breakpoint at 2  $\mu\text{g/ml}$  could be temporarily proposed for NTM's Bdq susceptibility testing, including both RGM and SGM species. According to this breakpoint, the Bdq resistance rates of the six most prevalent species were 5.6%(2/36), 0%(0/22), 2.2%(1/45), 10.0%(1/10), 10.0%(4/40), and 6.1%(2/33) for *M. intracellulare*, *M. avium*, *M. kansasii*, *M. gordonae*, *M. abscessus*, and *M. fortuitum*, respectively. Further validation about this breakpoint is needed to justify its proposal.

No recommended breakpoint of NTM species against Dlm has been proposed previously. The tentative ECOFFs defined in our assay for *M. kansasii* was 0.125  $\mu\text{g/ml}$ , and ECOFFs for other species were not proposed due to a small number of isolates or disappointing inhibitory activity. Dlm is administered at the dosage of 100 mg twice daily, i.e., at the lower limit of dosages with proven activity. With this dosage, the average 2-h postdosage plasma concentration was 0.39  $\mu\text{g/ml}$  among healthy volunteers administered with Dlm alone (20). Based on the pharmacokinetic data and the MIC outcomes acquired in ours and other studies, we tentatively propose a breakpoint for the Dlm susceptibility testing at  $>0.25 \mu\text{g/ml}$ . According to this breakpoint, the Dlm resistant rates of the four most prevalent SGM species were 97.2% (35/36), 86.4% (19/22), 13.3% (6/45), and 40.0% (4/10) for *M. intracellulare*, *M. avium*, *M. kansasii*, and *M. goodii*, respectively. Dlm may be usable for some SGM species infection treatment but not very promising for RGM infection treatment.

Dlm is intracellularly activated by F420-dependent mycobacterial nitroreductase encoded by the gene *Rv3547* (*ddn*), and mutations in this gene could lead to Dlm resistance among clinical isolates of *M. tuberculosis* (21, 22). In contrast with other genes (*fgd1*, *fbIA*, *fbIB*, and *fbIC*) reported to be related with Dlm resistance, a mutation within *ddn* owned the highest frequency (29%) of Dlm resistance (21, 23). In this study, isolates of *M. avium* demonstrated high susceptibility variations within species against Dlm (Fig. 2). Thus, we analyzed *ddn* sequences of the probable susceptible isolates and probable resistant isolates. One *M. avium* isolate with an MIC of  $>32 \mu\text{g/ml}$  harbored a Thr79Arg mutation and Ser86Cys mutation simultaneously. According to the crystal structure of a Ddn protein of *M. tuberculosis* (PDB accession number 3R5L), Thr79 and Ser86 were located in  $\beta 5$ , which interacted with  $\beta 4$  and forms the larger sheet at the base of the structure. This beta barrel-like structure is probably involved in binding hydrophobic Dlm; nevertheless, this molecular model-based speculation needs to be verified by more laboratory experiments in the future. Furthermore, a study found that a mutation located at Leu107 in  $\beta 5$  caused Dlm resistance in *M. tuberculosis* (21). Thus, mutations within the sheet are probably associated with Dlm resistance. In our study, only 1 out of 13 isolates of *M. avium* with MIC of  $\geq 32 \mu\text{g/ml}$  had a *ddn* homologous gene mutation. Hence, *ddn* homologues might not be the only target for Dlm to explore its bacteriostatic activity.

Our study had several limitations. Most notably, the small sample size can cause bias. Thus, the proposed ECOFFs might change slightly with increasing sample size. Second, given that Bdq and Dlm had never been used before the strain collecting, all these strains were considered "wild type." Further studies, including strains that had been exposed to the given drugs are needed for defining appropriate breakpoints of NTM DST. Furthermore, we only used the reference strain H37Rv (ATCC 27294) as a drug-susceptible control for the broth micro dilution method, which is recommended as a "pan-susceptible" control organism by Clinical and Laboratory Standards Institute (CLSI), whereas no resistant-control strain was recommended by CLSI and used in this study.

In conclusion, this study demonstrated that Bdq had high *in vitro* activities against the overwhelming majority of NTM species, including RGM and SGM. Dlm presented inhibitory activity against certain SGM species; however, the susceptibility variations within the same species highlight the requirement of a susceptibility screening before drug administration. According to the tentative ECOFF data in our assay and pharmacokinetic data from other reports, 2  $\mu\text{g/ml}$  could be temporarily proposed as breakpoint for NTM susceptibility testing for Bdq. Our study provided important insights on clinical therapy of Bdq and Dlm for the treatment of NTM infections.

## MATERIALS AND METHODS

**Ethics statement.** As the study only concerned laboratory testing of mycobacteria without the direct involvement of human subjects, ethics approval was not sought.

**Reference strains and clinical isolates.** All the mycobacterial reference strains stored in the Bio-bank in Beijing Chest Hospital (Beijing, China) were tested against Bdq and Dlm *in vitro*, including 33



rapidly growing mycobacterium (RGM) and 19 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 are listed in Table 1. A total of 209 NTM clinical isolates, collected between 2015 and 2016 in Beijing Chest Hospital, were recruited, including 40 *M. abscessus*, 33 *M. fortuitum*, 36 *M. intracellulare*, 22 *M. avium*, 45 *M. kansasii*, and 10 *M. goodii* isolates. The species constitution of the remaining 23 isolates is presented in Table 2. All the 209 NTM clinical strains were isolated from tuberculosis-suspected patients. The strains were classified as NTM preliminarily with *p*-nitrobenzoic acid-containing medium and then were differentiated into species level by 16S rRNA, *hsp65*, *rpoB*, and 16-23S rRNA internal transcribed spacer sequencing (9). All the isolates were stored at  $-80^{\circ}\text{C}$  and subcultured on Lowenstein-Jensen (LJ) medium before performing the microplate alamarBlue assay (MABA).

**MIC determination.** Bdq and Dlm were purchased from Liye-Pharmaceutical (Nanjing, China) and Shanghai Biochempartner Co., Ltd. (Shanghai, China), respectively. Both drugs were dissolved in dimethyl sulfoxide (DMSO). Stock solutions were aseptically prepared at concentrations of 2 mg/ml. The broth microdilution method was performed according to guidelines of CLSI (24). 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 medium. The broth microdilution format was set up as 2-fold dilution, and the concentration for Bdq ranged from 0.0078  $\mu\text{g/ml}$  to 32  $\mu\text{g/ml}$ , while for Dlm the concentration ranged from 0.125  $\mu\text{g/ml}$  to 32  $\mu\text{g/ml}$ . Briefly, a bacterial suspension of 0.5 McFarland standard was prepared and then diluted and inoculated into a 96-well microtiter plate to achieve a final bacterial load at  $10^5$  CFUs per well. Plates were then incubated at  $37^{\circ}\text{C}$  for 7 days for SGM and 3 days for RGM, respectively. A 70- $\mu\text{l}$  solution containing 20  $\mu\text{l}$  alamarBlue and 50  $\mu\text{l}$  Tween 80 (5%) was added to each well and incubated for 24 h at  $37^{\circ}\text{C}$  before assessing color development. A change from blue to pink or purple indicated bacterial growth (25). 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 excellent inhibitory activity demonstrated by Bdq/Dlm, the ECOFF was determined according to the distribution profile of the MIC values. For unimodal MIC distribution profile, ECOFF was defined as the concentration that could inhibit  $>95\%$  of the bacterial population; for bimodal MIC distribution profile, ECOFF was set between the two populations.

**Ddn encoding gene sequencing and protein alignment.** The aerobic killing mechanism of Dlm involves the inhibition of cell wall mycolic acid biosynthesis. This reaction is catalyzed by deazaflavin (F420)-dependent nitroreductase (Ddn), which is encoded by Rv3547 in *M. tuberculosis* (12). Mutations in *ddn*, causing the inhibition of cell wall mycolic acid biosynthesis, result in DLM resistance (21, 26). Sequence analysis of the *ddn* gene of the *M. avium* isolates was performed. The primers used to sequence *ddn* in *M. avium* was as follows: forward, 5'-GATCATCACCGACCGACA-3'; and reverse, 5'-TTCGACCGCCGCAATGAACG-3'. The *ddn* gene of the reference strains of the four most isolated species plus *M. tuberculosis* were also sequenced, and mutation was defined in contrast with the sequences of the reference strains. The amplification products were sequenced by Tsingke Company (Beijing, China). Multiple sequence alignment of the homologous proteins was performed using the Clustal Omega software. Structure-based multiple sequence alignment was performed with ESPript 3 based on the crystal structure of Rv3547 protein of *M. tuberculosis* from <http://esprpt.ibcp.fr/ESPript/ESPript/>.

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