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Primary Clofazimine and Bedaquiline Resistance among Isolates from Patients with Multidrug-Resistant Tuberculosis

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ABSTRACT Clofazimine has been repurposed for the treatment of tuberculosis, especially for multidrug-resistant tuberculosis (MDR-TB). To test the susceptibility to clofazimine of *Mycobacterium tuberculosis* clinical isolates, MICs of clofazimine were determined using the microplate alamarBlue assay (MABA) method for 80 drug-resistant isolates and 10 drug-susceptible isolates for comparison. For five clofazimine-resistant strains isolated from previously treated pre-extensively drug-resistant TB (pre-XDR-TB) and XDR-TB patients without prior exposure to clofazimine or bedaquiline, clofazimine MICs were ≥1.2 μg/ml. Four isolates with cross-resistance to bedaquiline had *Rv0678* mutations. The other isolate with no resistance to bedaquiline had an *Rv1979c* mutation. This study adds to a recent study showing that 6.3% of MDR-TB patients without prior clofazimine or bedaquiline exposure harbored isolates with *Rv0678* mutations, which raises concern that preexisting resistance to these drugs may be associated with prior TB treatment. Furthermore, we propose a tentative breakpoint of 1.2 μg/ml for clofazimine resistance using the MABA method. More-widespread surveillance and individualized testing for clofazimine and bedaquiline resistance, together with assessment of their clinical usage, especially among previously treated and MDR-TB patients, are warranted.

KEYWORDS *Mycobacterium tuberculosis*, clofazimine, bedaquiline, resistance, *Rv0678*

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The emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) poses a great challenge for the control of tuberculosis worldwide. The treatment of patients with MDR-TB and XDR-TB has often failed owing to a lack of effective drugs (1). Clofazimine, a riminophenazine drug, was originally described as having antimycobacterial activity in 1957 (2). It has been commonly used in the treatment of leprosy since 1962 (3). Recently, there has been renewed interest in the potential use of clofazimine to treat MDR-TB and XDR-TB and to shorten tuberculosis treatment (4–6). The introduction of clofazimine to treat tuberculosis should be accompanied by the generation of drug susceptibility data for relevant clinical isolates in order to reduce the risk of inappropriate treatment, early development of drug resistance, and transmission of resistant strains. Therefore, knowing the MIC distribution of clofazimine is important because clofazimine is now recommended by the WHO as a component of the new short-course MDR-TB regimen (7).

Redox cycling of clofazimine following reduction by NADH:quinone oxidoreductase of *Mycobacterium tuberculosis* and the resultant production of reactive oxygen species is one apparent mechanism by which clofazimine exerts bactericidal effects (8). How-

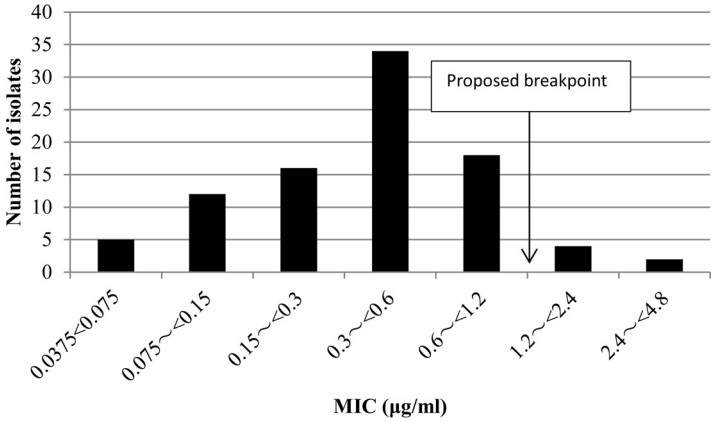

FIG 1 Distribution of clofazimine MICs determined by the MABA method for 90 *M. tuberculosis* clinical isolates.

ever, the mechanisms of resistance to clofazimine in *M. tuberculosis* are still incompletely characterized. Recently, mutations in *Rv0678*, which encodes the MarR-like transcriptional regulator of the MmpS5-MmpL5 efflux system, were shown to cause cross-resistance between clofazimine and bedaquiline *in vitro* (9, 10). Indeed, mutations in *Rv0678* were the major mechanism of clofazimine resistance among isolates selected with clofazimine *in vitro* (9, 11). During clinical trials of bedaquiline, isolates with reduced susceptibility to bedaquiline were shown to have *Rv0678* mutations and cross-resistance to clofazimine (10, 12). Recently, bedaquiline susceptibility testing identified a bedaquiline-resistant isolate with a heterogeneous *Rv0678* mutation profile isolated from a new MDR-TB case without prior exposure to bedaquiline or clofazimine (13). However, the prevalence of clofazimine-resistant strains and mutations in *Rv0678* in clinical *M. tuberculosis* isolates remains unknown. Mutations in the putative proline aminopeptidase gene *pepQ* (*Rv3525c*) have also been shown to confer low-level cross-resistance between bedaquiline and clofazimine *in vitro* and in mice (14) but have yet to be demonstrated among clinical isolates. The objectives of our study were (i) to determine the distribution of clofazimine MICs against clinical isolates and (ii) to investigate the possible mechanism of clofazimine resistance among isolates with reduced susceptibility.

RESULTS

MIC distribution of clofazimine in clinical isolates. The susceptibility of the quality control strain H37Rv was determined once with each batch of clinical isolates, and reproducible results were generated in this study, with MIC values of clofazimine between 0.14 and 0.28 μg/ml ($n = 6$, geometric mean = 0.24 μg/ml). The 90 study isolates were recovered from clinical samples of tuberculosis patients originating from China. Seventy-eight point seventy-five percent of drug-resistant patients and 60% of drug-susceptible patients had previously received treatment for tuberculosis. Testing the isolates with doubling concentrations of clofazimine of between 0.0375 and 4.8 μg/ml, we observed a bell-shaped MIC distribution (Fig. 1). We identified five clofazimine-resistant strains for which MIC values were greater than 1.2 μg/ml. The five strains were selected for mutation analysis based on a previous study suggesting a clofazimine susceptibility testing breakpoint of 1 μg/ml using the MGIT 960 method (15).

Demographic characteristics of patients with clofazimine-resistant isolates. Clofazimine-resistant strains ($n = 5$) were isolated from five patients between 2012 and 2013 (Table 1). Three patients were diagnosed with pre-XDR tuberculosis, and the other two patients were diagnosed with XDR-TB (confirmed by genetic analysis [see Table S2 in the Supplemental Material]). All patients had been previously treated with antituberculosis drugs but had no documented exposure to clofazimine or bedaquiline. All

TABLE 1 Epidemiologic and clinical data of clinical *M. tuberculosis* isolates[b]

Isolate	Type	Drug resistance profile	Age (yr)	Gender	Geographic location	Yr of isolation
10149	XDR	INH, RIF, EMB, CAP, AMK, OFX, LVX, ETO	23	Male	Gansu	2012
10601	Pre-XDR	INH, RIF, STR, EMB, OFX, LVX, ETO	28	Female	Shanxi	2012
12657	Pre-XDR	INH, RIF, STR, EMB, OFX, LVX, ETO	53	Male	Hebei	2013
13476	XDR	INH, RIF, STR, EMB, OFX, LVX, CAP, AMK, ETO, PAS	27	Female	Jilin	2013
11873	Pre-XDR	INH, RFP, EMB, OFX, LVX	35	Male	Beijing	2013
16833[a]	XDR	INH, RIF, STR, EMB, OFX, LVX, CAP, AMK, ETO, PAS	43	Female	Beijing	2014

[a]Clofazimine-susceptible strain.
[b]The patients from whom the isolates were derived had all been treated for TB prior to our study. The genotype for every isolate was Beijing.

five patients lived in different places in China and did not have any known epidemiologic relationships. All five clofazimine-resistant strains were identified as the Beijing genotype by a multiplex PCR (details are provided in Fig. S1).

Cross-resistance between clofazimine and bedaquiline. To identify clofazimine-bedaquiline cross-resistance, we tested the MIC of bedaquiline against clofazimine-resistant strains using the microplate alamarBlue assay (MABA) method. Four of five clofazimine-resistant isolates were also resistant to bedaquiline. The other isolate, 11873, was susceptible to bedaquiline (Table 2). Fourteen clofazimine-susceptible isolates for which MICs were less than 1.2 μg/ml were also tested and confirmed to be susceptible to bedaquiline.

Genome analysis. As mutations in *Rv0678* are a major mechanism of clofazimine-bedaquiline cross-resistance (11) and several target-based resistance mutations in the *atpE* gene have been described in bedaquiline-resistant strains selected *in vitro* (16–18), we sequenced the *Rv0678* and *atpE* genes. All four clofazimine-bedaquiline cross-resistant strains had a mutation in the *Rv0678* gene. Strains from different patients had *Rv0678* gene mutations at different nucleotide positions. No *Rv0678* mutations were found in 14 tested clofazimine-susceptible strains. No *atpE* mutations were found in any strain.

To identify other mechanisms of clofazimine resistance in clofazimine-resistant isolates, we subjected all five clofazimine-resistant strains and a clofazimine-susceptible control strain (16833) to whole-genome sequencing. Strain 16833, isolated from a retreated XDR-TB patient, had a Beijing genotype without mutations in *Rv0678*, *Rv1979c*, *pepQ*, and *atpE* and was susceptible to both clofazimine and bedaquiline (Table 2). Comparative genome sequence analysis confirmed the *Rv0678* mutations previously identified by PCR-based sequencing in the four clofazimine-bedaquiline cross-resistant strains (strains 10149, 10601, 12657, and 13476) and the absence of mutations in clofazimine-susceptible strain 16833. There were no other common mutations found by single nucleotide polymorphism (SNP) analysis. Clofazimine-resistant strain 11873, which did not harbor any *Rv0678* mutations, had an a155c mutation in *Rv1979c* (Table 2). No mutations were found in *pepQ* among the clofazimine-resistant strains.

TABLE 2 Drug susceptibility profiles of clofazimine-resistant and wild-type *M. tuberculosis*

	MIC (μg/ml)		Mutation[a]			
Isolate	Clofazimine	Bedaquiline	*Rv0678*	*Rv1979c*	*Rv2535c*	*atpE*
10149	1.2	0.78	*t437c* (*M146T*)	WT	WT	WT
10601	4	0.73	*g5t* (*S2I*)	WT	WT	WT
12657	2.09	0.39	*c158t* (*S53L*)	WT	WT	WT
13476	4.16	1.54	*t350g* (*L117R*)	WT	WT	WT
11873	1.2	0.08	WT	*a155c* (*V52G*)	WT	WT
16833	0.14	0.1	WT	WT	WT	WT
H37Rv	0.24	0.1	WT	WT	WT	WT

[a]WT, wild type. Isolates were sequenced by whole-genome sequencing.

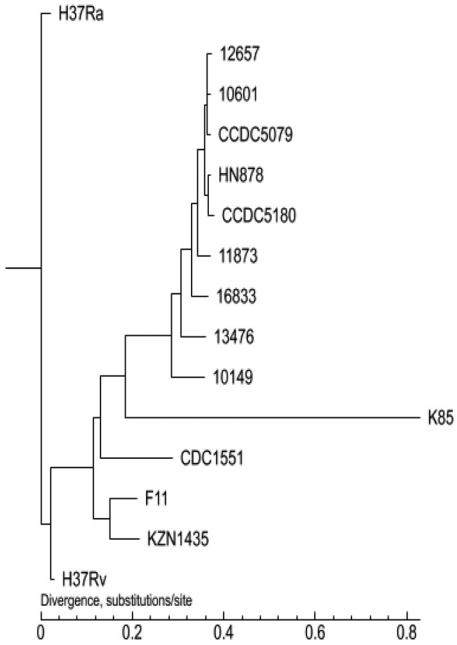

FIG 2 Phylogenetic analysis of *M. tuberculosis* isolates. The tree was constructed by TreeBeST using the PhyML method, set to 1,000 bootstrap resamplings.

Phylogenetic analysis of clofazimine-resistant isolates. To investigate the molecular evolution and genetic diversity of the clofazimine-resistant strains, maximum-likelihood phylogenetic trees were constructed based on SNPs from whole-genome sequences of the 5 clofazimine-resistant isolates, 1 clofazimine-susceptible isolate, and 9 other completely sequenced *M. tuberculosis* strains. The reliability of each node was estimated from 1,000 random bootstrap resamplings of the data. The phylogenetic relationships among the 5 clofazimine-resistant isolates were similar to those of the clofazimine-susceptible isolate in the phylogenetic trees (Fig. 2). The 6 newly sequenced clinical isolates as well as the 3 previously sequenced Beijing lineage strains CCDC5079, CCDC5180, and HN878 formed a single clade, which confirmed the genotype of these strains as determined by multiplex PCR (Fig. S1).

DISCUSSION

The generation of MIC data against relevant clinical isolates is necessary to design appropriate treatment regimens, prevent the occurrence of drug resistance, and reduce the transmission of resistant strains. The wild-type clofazimine MIC distribution of *M. tuberculosis* in 7H10 medium has been reported for 45 consecutive drug-susceptible clinical isolates from Sweden (19). The modal MIC was 0.25 μg/ml. For only one isolate was the MIC 0.5 μg/ml. Meanwhile, the MIC of clofazimine for the H37Rv strain in 7H10 medium was 0.125 μg/ml (19). A breakpoint concentration of 1 μg/ml for clofazimine susceptibility testing using the MGIT 960 method was proposed after study of 26 multidrug-resistant clinical isolates from the Netherlands revealed an MIC_{90} of 0.25 μg/ml (15). Our study determined clofazimine MIC values for 90 clinical isolates from China, mostly from patients with drug-resistant tuberculosis (80 isolates). Excluding five isolates with MICs of $\geq$1.2 μg/ml, MIC values ranged from 0.0375 to $<$1.2 μg/ml, with a mode at 0.6 μg/ml. All five clofazimine-resistant isolates had mutations in *Rv0678* ($n = 4$) or *Rv1979c*, whereas none of the 14 strains for which the MIC of clofazimine was below 1.2 μg/ml had mutations in *Rv0678* or *atpE*. One limitation is that we tested only 14, or approximately one-sixth, of the clofazimine-susceptible strains for the presence or absence of *Rv0678* mutations. Testing of a larger number of diverse strains from multiple laboratories with more-extensive reproducibility testing is needed to conclusively identify a breakpoint value for clofazimine with the MABA method, as well as to

fully understand the association of *Rv0678* mutations with elevated clofazimine MICs and the impact of elevated MICs on the clinical response to clofazimine. Nevertheless, in light of the genotypic results, our MABA results may set a tentative MABA breakpoint value of 1.2 μg/ml for reduced clofazimine susceptibility, consistent with the breakpoint suggested by the MGIT 960 method (15).

By the end of 2014, acquired resistance to clofazimine and bedaquiline in a patient with MDR-TB was reported (12). The isolate exhibited resistance to clofazimine (MIC $\geq$ 4 μg/ml) and to bedaquiline (MIC = 3.2 μg/ml), with a corresponding mutation in the *Rv0678* gene (GTG→GCG, leading to an M1A substitution) (20). Torrea et al. also found one *Rv0678* mutant for which the bedaquiline MIC was high from an MDR-TB patient without overt clofazimine exposure (13). More recently, Villellas et al. described *Rv0678* mutations in 6.3% of isolates from MDR-TB patients without prior clofazimine or bedaquiline exposure who were enrolled in bedaquiline treatment trials (21). We now report five clofazimine-resistant strains isolated from pre-XDR and XDR-TB patients in China between 2012 and 2013, despite no documented prior exposure of the patients to clofazimine or bedaquiline. One possible explanation is that *Rv0678* mutations are selected by treatment with other TB drugs. Villellas et al. found a higher frequency of *Rv0678* mutations among isolates from MDR-TB patients than among isolates from drug-susceptible TB patients (21). The same authors also found that *Rv0678* mutants were modestly less susceptible to rifampin (RIF), although the difference was not statistically significant. In addition, *Rv0676c* to *Rv0678* were found to be upregulated in a rifampin-resistant strain after exposure to rifampin *in vitro* (22). Thus, it is possible that MmpL5 (*Rv0676c*) and MmpS5 (*Rv0677c*) are involved in rifampin efflux out of the *M. tuberculosis* cell. Since *Rv0678* is a transcriptional repressor of MmpS5 and MmpL5, mutations in this gene lead to an increased expression of this efflux pump (23). Rv0678 shows a homology of 49.40% with the well-characterized MarR protein of *E. coli* (24). This family of regulatory proteins plays an important role in the development of antibiotic resistance (25). Besides conferring resistance to clofazimine and bedaquiline, mutations in *Rv0678* have previously been described to confer resistance to antifungal azoles (23) and to an oxazole with antituberculosis activity (26). A high proportion (60%) of patients contributing the drug-susceptible isolates for this study had prior treatment history. This may reflect that the isolates were obtained from referral laboratories from all around the country. Therefore, the proportion with clofazimine-bedaquiline cross-resistance may not be generalizable to a patient population without prior rifampin treatment. This suggests that regimens containing clofazimine and/or bedaquiline may still be applied to patients with drug-susceptible TB even if there is a higher proportion of *Rv0678* mutations among MDR-TB cases. Further study of the pathogenesis, prevalence, and significance of *Rv0678* mutations among MDR-TB isolates is urgently needed before bedaquiline and clofazimine use becomes more widespread.

Rv1979c is associated with isoniazid (INH) resistance (27). Mutations of *Rv1979c* were found in *in vitro*-selected clofazimine-resistant isolates without *Rv0678* mutations (11). It is of interest to note that the clofazimine-resistant strain studied here, 11873, had a mutation in *Rv1979c* and did not display cross-resistance to bedaquiline. Further studies need to verify the role of *Rv1979c*, which is annotated as a possible conserved permease that might be involved in amino acid transport. It is possible that *Rv1979c* is involved in clofazimine transport or uptake that alters the physiology of the bacteria (11).

Four of five clofazimine-resistant isolates had *Rv0678* mutations and demonstrated cross-resistance to bedaquiline. In addition to identifying one mutation, *c158t* (*S53L*), that was previously reported (11), we identified three new mutations scattered across the *Rv0678* gene (Table 2). Several target-based resistance mutations in the *atpE* gene have been described in bedaquiline-resistant strains previously selected *in vitro* (16–18), but we did not find any *atpE* mutations in these isolates. Almeida et al. (14) discovered mutations in *pepQ* that confer low-level resistance to both clofazimine (0.5 to 1 μg/ml) and bedaquiline (0.12 to 0.25 μg/ml) in *M. tuberculosis*, but we did not find *pepQ*

mutations in the five clinical isolates with clofazimine MICs of ≥1.2 μg/ml. Our study confirms and expands our understanding of mechanisms of resistance to clofazimine by identifying additional mutations in *Rv0678* that cause cross-resistance between clofazimine and bedaquiline, as well as demonstrates the presence of such mutants in Chinese pre-XDR-TB and XDR-TB patients without prior exposure to clofazimine or bedaquiline. We also provide additional evidence that mutations in *Rv1979c* may be associated with clofazimine resistance without being associated with cross-resistance to bedaquiline.

The five tuberculosis patients who produced clofazimine-resistant isolates lived in different places in China without any known epidemiological relationship. Whole-genome sequencing revealed that the phylogenetic classifications of the clofazimine-resistant isolates and the clofazimine-susceptible isolate were not different (Fig. 2). Though they all belonged to the Beijing genotype, we can conclude that the clofazimine-resistant isolates do not originate from the same strain. Our finding of isolates displaying spontaneous resistance to clofazimine and bedaquiline emphasizes the urgency of more-extensive surveillance for such resistance prior to more-widespread usage of these drugs and the pressing need for reliable and feasible drug susceptibility testing to aid in regimen selection for individual patients in order to prevent further selection and transmission of drug-resistant strains.

MATERIALS AND METHODS

Bacterial strains. Eighty drug-resistant strains of *M. tuberculosis* from 80 tuberculosis patients and 10 drug-susceptible strains for comparison from 10 tuberculosis patients, all collected between 2012 and 2014, were obtained for this study from the National Clinical Laboratory on Tuberculosis, Beijing Chest Hospital. Drug resistance is defined as resistance to any of the following drugs: isoniazid (INH), rifampin (RIF), streptomycin (STR), ethambutol (EMB), ofloxacin (OFX), levofloxacin (LVX), capreomycin (CAP), amikacin (AMK), ethionamide (ETO), and *p*-aminosalicylic acid (PAS), using the absolute-concentration method, with 0.2, 40, 10, 2, 2, 2, 40, 30, 40, and 1 μg/ml, respectively, as the critical concentrations on Löwenstein-Jensen (LJ) medium. The *M. tuberculosis* strains were grown in Middlebrook 7H9 broth (Difco, USA) supplemented with 0.2% (vol/vol) glycerol, 0.05% Tween 80, and 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) (Becton-Dickinson, USA).

MIC determination. Clofazimine (Biochempartner, China) and bedaquiline (Biochempartner, China) MICs were determined by the microplate alamarBlue assay (MABA), using 2-fold dilutions ranging from 9.6 to 0.0375 μg/ml and 3.2 to 0.0125 μg/ml, respectively (28, 29). *M. tuberculosis* (100 μl containing 2×10^5 CFU) was added to wells, yielding a final testing volume of 200 μl. The plates were incubated at 37°C; on day 7 of incubation, 12.5 μl of 20% Tween 80 and 20 μl of alamarBlue were added to all wells. After incubation at 37°C for another 16 to 24 h, the fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The MIC was defined as the lowest concentration eliciting a reduction in fluorescence of ≥90% relative to the mean fluorescence of replicate drug-free controls. *M. tuberculosis* H37Rv was used as a drug-susceptible control.

PCR and DNA sequencing. Genomic DNA from *M. tuberculosis* clinical isolates was extracted using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's protocol.

To amplify and sequence the *Rv0678* gene, forward primer *Rv0678*-F (5′-TGCCTTCGGAACCAAAGAA-3′) and reverse primer *Rv0678*-R (5′-GACAACACGGTCACCTACAA-3′) were used (11). The *atpE* gene was PCR amplified using primers *atpE*-F (5′-TGTACTTCAGCCAAGCGATGG-3′) and *atpE*-R (5′-CCGTTGGGAAT GAGGAAGTTG-3′) (16), and the PCR products were sent to Rui Biotech Company for sequencing.

Whole-genome sequencing. *M. tuberculosis* clinical isolates were subjected to genomic DNA extraction as described above.

DNA samples were quantified, subjected to quality control measures, and visualized with a Qubit fluorometer and an agarose electrophoresis gel. Paired-end sequencing libraries were constructed with insert sizes of approximately 300 bp using standard kits from Illumina according to the manufacturer's instructions.

Genomic DNA was sequenced using an Illumina Hiseq4000 (Illumina, Inc.). For each strain, 1.3 to 1.8 gigabases (230-fold to 350-fold genome coverage) were generated after low-quality raw reads were discarded. Image analysis and base calling were done using the Illumina GA Pipeline software. Genome assembly was performed using SOAPdenovo v2.04 software, with clean short reads. Reads were aligned with the reference sequences of *M. tuberculosis* H37Rv (GenBank accession number NC_000962.3) and clinical isolate 16833 using SOAPaligner v2.21. Whole-genome alignments for comparative analyses were generated using MUMmer v3.22. The maximum-likelihood phylogenetic trees were constructed by TreeBeST v1.9.2 using the PhyML method, based on SNPs from whole-genome sequences.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00239-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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