Clofazimine exposure *in vitro* selects efflux pump mutants and bedaquiline resistance

Nabila Ismail[1#], Remco P.H Peters [1, 2], Nazir A. Ismail [1, 3, 4], Shaheed V. Omar [4]

[1]Department of Medical Microbiology, Faculty of Health Sciences, University of Pretoria, Pretoria, 0001, South Africa

[2]Department of Medical Microbiology, School for Public Health and Primary Care (CAPRHI), Maastricht University, Maastricht, 6200 MD, The Netherlands

[3]Faculty of Health Sciences, University of the Witwatersrand and the National Health Laboratory Service, Johannesburg, South Africa

[4]Centre for Tuberculosis, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, 2192, South Africa

[#]Corresponding author: Nabila Ismail, e-mail: nabilai@nicd.ac.za

Abstract

Six in-vitro clofazimine-resistant spontaneous mutants obtained from a wild-type or pyrazinamide-resistant ATCC reference strain were selected to evaluate bedaquiline cross-resistance. The reverse was conducted for bedaquiline mutants. All clofazimine mutants harbouring an *rv0678* mutation displayed phenotypic cross-resistance. We observed the same for *rv0678* bedaquiline mutants, however *atpE* bedaquiline mutants showed no phenotypic cross-resistance. This confirms that upfront clofazimine usage may impact subsequent bedaquiline use due to a shared efflux resistance pathway.

Keywords

Clofazimine, Bedaquiline, Efflux pump mutants, Cross-resistance

Running title

Efflux pump cross-resistance tuberculosis

Clofazimine has recently been incorporated into the WHO shortened drug-resistant TB (DR-TB) regimens due to recent evidence of good efficacy (1, 2). Patients with rifampicin-resistant or MDR-TB can either be placed on the shortened DR-TB regimen containing clofazimine or on a regimen comprising both bedaquiline (WHO Group A) and clofazimine (WHO Group B) if possible (3). The *rv0678* gene mutation has been identified in mutants and clinical isolates with increased clofazimine MIC values (4, 5). Although bedaquiline's primary drug target is the adenosine triphosphate (ATP) synthase (encoded by *atpE* gene), an enzyme involved in the synthesis of ATP (6), mutations in the *rv0678* gene have also been found to result in bedaquiline resistance. This *rv0678* gene encodes the *mmpR5* repressor protein, with *rv0678* mutations resulting in *mmpL5-mmpS5* efflux pump overexpression (7). Mutations in the *rv1979c* and *rv2535c* (*pepQ*) genes have also been found in clofazimine-resistant mutants (4). In clinical isolates with prior bedaquiline exposure, *rv0678* mutations were causative factors for increased clofazimine MIC values (8).

As the occurrence of cross-resistance between clofazimine and bedaquiline may reduce available treatment options, we aimed to determine if upfront clofazimine use could potentially result in increased bedaquiline MIC values due to mutations in a shared efflux pathway.

We isolated spontaneous mutant colonies using 4× proposed critical concentration (CC) of clofazimine (1 µg/mL, (9)) with a Luria-Delbrück fluctuation assay as described previously (10). Two *M. tuberculosis* American Type Culture Collection (ATCC) reference strains were used: ATCC27294 (H37Rv), a wild type/fully susceptible strain [WT] (ATCC27294) and a pyrazinamide-resistant [PZA^R] strain (ATCC35828). The latter was used for its potentially higher propensity for mutation development as shown in our previous experiments (10) and because pre-existent PZA resistance in rifampicin-resistant and DR-TB is common (11, 12). Putative mutant colonies were sub-cultured for a single drug-free passage in preparation for clofazimine MIC testing (Range of 0.06- 4 µg/mL) using the MGIT960 platform (Becton Dickinson Diagnostic Systems (BD Biosciences), Sparks, Maryland, USA) (13). Cross-resistance to bedaquiline was evaluated in triplicate using MGIT960 (Range: 0.12- 8 µg/mL). Using the same methodology as described above, we evaluated clofazimine cross-resistance using six bedaquiline-resistant spontaneous mutants derived from the same ATCC strains and a bedaquiline CC of 1 µg/mL, (9).

DNA Extraction was performed using the generic protocol on the NucliSENS easyMAG (BioMérieux) and Whole Genome Sequencing (WGS) was carried out on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the Nextera XT DNA library kit. Single nucleotide polymorphisms (SNPs) or insertions/deletions (indels) were detected at a frequency of >30% (14) and a Phred score of ≥Q20 (≥99% accuracy), exploring the *rv0678*, *atpE*, *rv1979c* and *rv2535c* genes targets only.

From a multitude of putative clofazimine-resistant spontaneous mutants, three from the PZA^R strain and three from the WT strain, having MIC values ranging between 1-4 µg/mL, were randomly selected (Table 1). All of these harboured *rv0678* mutations and phenotypic bedaquiline cross-resistance (MIC: 4-8 µg/mL).

Table 1: Clofazimine-resistant spontaneous mutants with associated bedaquiline phenotypic data. Novel mutations (not previously identified in literature) are in bold text.

ATCC strain		CFZ Mutant MIC (µg/mL)	BDQ MIC (µg/mL) n=3			Gene	
						atpE	*rv0678*
1	WT	2	8	8	8	WT	193delG (Ile67fs)
2	WT	4	8	8	8	WT	193delG (Ile67fs)
3	WT	4	8	8	8	WT	**A65T (Gln22Leu)**
4	PZA^R	1	4	8	8	WT	**T407C (Leu136Pro)**
5	PZA^R	2	8	8	8	WT	C214**T** (Arg72**Trp**)
6	PZA^R	4	4	8	8	WT	A97G (Thr33Ala)

Footnote:

Mutant and cross-resistance MGIT960 MIC values are described.

ATCC Strains: WT (wild-type/fully susceptible), PZA^R (pyrazinamide resistant)

NT: Nucleotide; AA: Amino Acid

Six randomly selected putative bedaquiline mutant colonies (five from the PZA^R strain and a single colony from the WT strain) were confirmed as phenotypically bedaquiline-resistant (MIC range: 4- >8 µg/mL, Table 2). Three bedaquiline-resistant mutants (*atpE* mutations only) showed no clofazimine cross-resistance (MIC range: 0.25- 0.5 µg/mL). Three mutants with both *atpE* and *rv0678* mutations displayed clofazimine cross-resistance (MIC range: of 2-4 µg/mL). From these, one mutant had

no detectable *rv0678* mutations at the 30% nor 10% frequency threshold for variant calling (Table 2). *Rv0678* Sanger sequencing (4) of DNA extracted from these mutants revealed a Ser63Gly mutation, previously implicated in bedaquiline resistance (15-18).

Table 2: Bedaquiline-resistant spontaneous mutants with associated clofazimine phenotypic data. Novel mutations (not previously identified in literature) are in bold text.

ATCC strain		BDQ Mutant MIC (µg/mL)	CFZ MIC (µg/mL) n=3			Gene	
						atpE	***rv0678***
1	WT	>8	0.25	0.25	0.5	A83T (Asp28Val)	WT
2	PZA^R	4	4	>4	>4	WT	**C403G (Arg135Gly)**
3	PZA^R	8	2	4	4	A83G (Asp28Gly)	A187G(Ser63Gly)*
4	PZA^R	>8	0.5	0.5	0.5	G187C (Ala63Pro)	WT
5	PZA^R	4	0.25	0.5	0.5	A83G (Asp28Gly)	WT
6	PZA^R	4	4	4	4	WT	C189A(Ser63Arg)

Footnote:

Mutant and cross-resistance MGIT960 MIC values are described.

ATCC Strains: WT (wild-type/fully susceptible), PZA^R (pyrazinamide resistant)

NT: Nucleotide; AA: Amino Acid

*Identified in triplicate clofazimine cross-resistance tubes using Sanger sequencing

In this study, cross-resistance resulted from four novel (Gln22Leu, Leu136Pro, Arg72Trp, Arg135Gly) and three previously described *rv0678* mutations (Ile67fs (4), Ser63Arg (7) and Thr33Ala (8)), all scattered across the *rv0678* gene (Figure 1). The Thr33Ala mutation was previously reported in a bedaquiline-treated clinical isolate with resultant clofazimine cross-resistance (8). Here, we show a WT strain exposed to clofazimine obtained this mutation and displayed bedaquiline cross-resistance, confirming the key role of *rv0678* mutations in cross-resistance. The *atpE* mutations (Asp28Gly, Asp28Val and Ala63Pro) observed have been previously described and occur in hotspot regions. No *rv1979c* or *rv2535c* (*pepQ*) mutations were identified. The occurrence of *rv0678* mutations due to clofazimine exposure appears to be the determining factor for resultant bedaquiline cross-resistance and *vice versa*.

Therefore, irrespective of the drug used for resistance selection, the common efflux-based pathway used by these drugs is highlighted through this study (19).

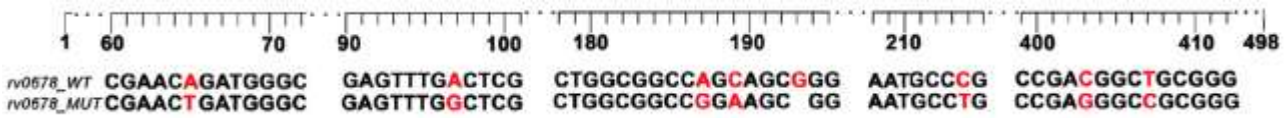

Figure 1: Mutations in *rv0678* gene resulting in bedaquiline and clofazimine cross-resistance. Ellipses indicate regions of the gene not shown. Single nucleotide polymorphisms are indicated in red as a change from the wild-type sequence (*rv0678*_WT) to the mutated sequence (*rv0678*_MUT).

Utmost care was taken to select single colonies but smaller colonies may have combined resulting in heterogeneous populations with both *atpE* and *rv0678* mutations. However, these populations confirmed the role of *rv0678* mutations further and notably have been previously described in a single clinical isolate (20). We were only able to obtain a single bedaquiline-resistant mutant from the WT strain but we assume that obtaining further bedaquiline-resistant mutants from the WT strain would not impact our findings.

The replacement of injectable drugs for bedaquiline supports outcome improvement (21), while combination therapy with bedaquiline and clofazimine mitigates against emergent cross-resistance and allows benefits of both drugs to be realised early on. With the current roll out of the shorter DR-TB regimen possible cross-resistance could occur following exposure to a single drug (clofazimine), resulting in subsequent bedaquiline loss or *vice versa*. It remains enigmatic whether efflux-pump inhibitors can overcome the presence of an efflux-based resistance pathway (*rv0678* mutations).

In this study, we show that bedaquiline-naïve reference strains can accumulate resistance to bedaquiline following clofazimine exposure due to mutation of the *rv0678* gene. Clofazimine cross-resistance can only develop in the presence of *rv0678* but not *atpE* mutations. As *rv0678* mutations play a role in resistance to both these drugs, there is an important emergent need to catalogue *rv0678* mutations with associated phenotypic data, which could steer usage. Phenotypic drug susceptibility testing and targeted *rv0678* sequencing (where feasible) should be considered as part of

diagnostic algorithms to rule out cross-resistance and ensure optimal treatment outcomes.

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Data availability. Raw sequence data are accessible on the NCBI platform under accession no. PRJNA494324.

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