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Emergence of canonical and non-canonical genomic variants following *in vitro* exposure of clinical *Mycobacterium tuberculosis* strains to bedaquiline or clofazimine

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Abstract

In *Mycobacterium tuberculosis*, bedaquiline and clofazimine resistance occurs primarily through *Rv0678* variants, a gene encoding a repressor protein which regulates *mmpS5*/*mmpL5* efflux pump gene expression. Despite the shared effect of both drugs on efflux, little else is known about other pathways affected. We hypothesized that *in vitro* generation of bedaquiline- or clofazimine-resistant mutants could provide insight into additional mechanisms of action. We performed whole genome sequencing and determined phenotypic minimal inhibitory concentrations for both drugs on progenitor and mutant progenies. Mutants were induced through serial passage on increasing concentrations of bedaquiline or clofazimine. *Rv0678* variants were identified in both clofazimine- and bedaquiline-resistant mutants with concurrent *atpE* SNPs occurring in the latter. Of concern, was the acquisition of variants in the F420 biosynthesis pathway in clofazimine-resistant mutants obtained from either a fully susceptible (*fbiD*: del555GCT) or rifampicin mono-resistant (*fbiA*: 283delTG and T862C) progenitor. The acquisition of these variants possibly implicates a shared pathway between clofazimine and nitroimidazoles. Pathways associated with drug tolerance and persistence; F420 biosynthesis; glycerol uptake and metabolism; efflux and NADH homeostasis appear to be affected following exposure to these drugs. Shared genes affected for both drugs include *Rv0678*, *glpK*, *nuoG* and *uvrD1*. Genes with variants in the bedaquiline resistant mutants included *atpE*, *fadE28*, *truA*, *mmpL5*, *glnH* and *pks8*, while clofazimine resistant mutants displayed *ppsD*, *fbiA*, *fbiD*, *mutT3*, *fadE18*, *Rv0988* and *Rv2082* variants. These results show the importance of epistatic mechanisms as a means of responding to drug pressure and highlight the complexity of resistance acquisition in *M. tuberculosis*.

Introduction

Bedaquiline and clofazimine, are novel and repurposed anti-tuberculosis (TB) drugs respectively, which offer promising options to treat and alleviate the TB disease burden. In particular, they are used as therapeutics for drug-resistant TB, which is more challenging to diagnose and treat compared to susceptible disease forms. Bedaquiline and clofazimine usage has increased since the WHO approved both drugs for the treatment of rifampicin-resistant tuberculosis (TB) [1]. At a juxtaposition to this increased usage is the lack of a rapid genotypic drug susceptibility test (DST) for these two drugs [2]. Although variants in the *Rv0678* gene encoding for a repressor protein, which affects the expression of the *mmpS5/mmpL5* efflux pump [3-5], are associated with both clofazimine and bedaquiline resistance, there is a lack of understanding of all genetic components involved in resistance for these two drugs. This is due to the rarity of resistant *Mycobacterium tuberculosis* isolates available for investigation and the lack of agreement and availability of phenotypic DST associated with genotypic DST data [6]; the presence of both wild-type and variant forms of *Rv0678* (heteroresistance) observed in clinical isolates [7]; and the inconsistency of the association of insertions and deletions (indels) in *Rv0678* and bedaquiline resistance [8].

While bedaquiline is known to target subunit C of the ATP synthase, encoded by *atpE*, there are a limited number of clinical strains with *atpE* variants [9]. Other non-target genes for bedaquiline include *Rv1979c* (a putative permease) and *pepQ* (cytoplasmic peptidase) [10]. Clofazimine resistance has been loosely associated with the latter two genes [3, 10]. Furthermore, clofazimine has been shown to be reduced enzymatically by the NADH:quinone oxidoreductase (encoded by *ndh2*), but to date no clofazimine-resistant

ndh mutants (or mutants with genetic variants in redox pathways) have been reported [11, 12]. In the 2021 WHO catalogue of drug resistance associated mutations, *Rv0678*, *pepQ*, *mmpS5* and *mmpL5* genes are considered to be tier 1: meaning that these genes are considered to most probably contain resistance conferring variants for both bedaquiline and/or clofazimine [6]. *Rv1979c* is a tier 2 gene, which has a reasonable probability of containing resistance conferring variants [6].

In this study, we aimed to identify whether *in vitro* exposure of a set of progenitor clinical *M. tuberculosis* isolates to either bedaquiline or clofazimine leads to the accumulation of variants in addition to *Rv0678* variants. We investigated the phenotypic and genotypic characteristics of mutants compared to the baseline characteristics of the progenitor strains.

Methods

All experimental work was done in BSL3 laboratory of the National TB Reference Laboratory and WHO TB Supranational Reference Laboratory (South Africa). The use of deidentified clinical *M. tuberculosis* strains was approved by The Research Ethics Committee (University of Pretoria, Faculty of Health Sciences- REF: 309/2016). The clinical isolates were collected during routine surveillance with drug-susceptibility and, in some cases, spoligotyping data. Figure 1 describes the experimental workflow for clinical sample set selection, *in vitro* mutant generation and global genomic analysis.

Sample set selection

Six clinical strains that belonged to either T-type, LAM, X-type and Beijing lineages and which had different drug-susceptibility profiles (fully susceptible, rifampicin-mono

resistant or multi-drug resistant) were selected. An ATCC27294 *M. tuberculosis* H37Rv reference strain was also included as a control strain. Each strain was cultured using the BACTEC MGIT960 automated liquid culture system (Becton Dickinson Diagnostic Systems (BD Biosciences), Sparks, Maryland, USA). This was followed by purity determinations (blood agar and ZN staining), baseline whole genome sequencing (WGS) and baseline susceptibility testing for bedaquiline and clofazimine in MGIT media [13, 14]. Strains used were abbreviated according to their susceptibility profiles; S (susceptible), rifampicin-mono resistant (R) and multi-drug resistant (M). The strains were S1 (referring to the Beijing susceptible strain), S2 (referring to the T-type susceptible strain), R1 (referring to the Beijing rifampicin-mono resistant strain), M1 (referring to the LAM MDR strain), M2 (referring to the T-type MDR strain) and M3 (referring to the X-type MDR strain).

Mutant generation

Bedaquiline- and clofazimine-resistant *M. tuberculosis* mutants were generated as previously described [13]. Briefly, bacterial cell suspensions of actively growing isolates with a turbidity equivalent to a McFarland 1.0 standard were inoculated on five Middlebrook 7H10 agar plates (supplemented with OADC) with different bedaquiline (Janssen Therapeutics, Titusville, NJ, USA) concentrations (range of 0.004-0.06 µg/ml) and a drug-free control plate. For the second passage, growth from the plate with the highest bedaquiline concentration was used to inoculate (McFarland 1.0 cell suspension) four plates (a drug-free control plate, a plate containing the growth-permitting concentration and plates containing either two- or four-fold higher bedaquiline concentrations). Passaging was continued for a total of five passages after which

confluent growth was scraped off from the plates and used for subsequent minimal inhibitory concentration (MIC) determinations. No further passaging was performed on plates containing >4 µg/ml bedaquiline. Clofazimine-resistant mutants were created using the same methodology using a starting range of 0.125-0.5 µg/ml clofazimine (C8895, Sigma-Aldrich Co., St Louis, USA). Passaging was continued for a total of four passages. For each passage, plates were incubated at 37°C until sufficient growth appeared for the creation of a McFarland 1.0 cell suspension (minimum 21 and maximum 28 days).

Baseline and mutant phenotypic characterization

Growth from MGIT tubes sub-cultured from each clinical isolate was used for MIC determinations and DNA extraction (WGS) for baseline characterization. Ten microliters of a suspension created from confluent growth from either the plates containing the highest drug concentration from the final passage or the last control passage were respectively inoculated into MGIT tubes for a drug-free passage to prepare the inoculum for subsequent MIC determinations using the BACTEC MGIT960 platform and DNA extraction.

Determination of minimal inhibitory concentration values

Bedaquiline (Janssen Therapeutics, Titusville, NJ, USA) and clofazimine (REF: C8895, Sigma-Aldrich Co., St Louis, USA) were formulated in DMSO (REF: 41639, Sigma-Aldrich Co.) to stock concentrations of 1 mg/ml and maintained at −20 °C (max: 3 months). Two-fold dilutions with a final concentration ranging from 8 to 0.125 µg/ml (bedaquiline) and 4 to 0.06 µg/ml (clofazimine) were prepared from the stock solutions. An 8 µg/ml clofazimine concentration could not be included as the color of the drug solution interfered with the

114 florescent detection of the BACTEC MGIT960 instrument. Minimal inhibitory
115 concentration (MIC) determinations were performed as previously described [13, 14]. A
116 1:5 dilution of a three- to five-day positive liquid culture was used to inoculate (500 µl)
117 seven MGIT tubes containing the above-described range of bedaquiline or clofazimine
118 concentrations. A further 1:100 dilution of the 1:5 suspension was used to inoculate (500
119 µl) a drug-free MGIT control tube. A H37Rv strain was included in each batch of
120 bedaquiline and clofazimine MIC determinations conducted. Tubes were incubated until
121 the growth control reached 400 growth units (GUs) or for a maximum of 28 days. The
122 MIC value was defined as the lowest drug concentration at which bacterial growth was
123 inhibited [15].

124 *Baseline and mutant genotypic characterization*

125 Genomic DNA extraction was performed using the on-board generic protocol on the
126 NucliSENS® easyMag® (BioMérieux, Marcy-l'Étoile, France). DNA concentrations were
127 determined was measured using a Qubit® 2.0 fluorometer (Life technologies, Carlsbad,
128 CA, USA) with the Qubit dsDNA High Sensitivity (HS) Assay kit (Life technologies).
129 Paired-end libraries were prepared using the Nextera XT DNA library kit (Illumina, San
130 Diego, CA, USA) according to manufacturer's protocol with a modified library
131 normalization step [16]. WGS was carried out using an Illumina MiSeq 2× 300bp V3
132 cartridge on the Illumina MiSeq platform.

133 Raw sequence data were analysed as previously described [17]. Briefly, reads were
134 trimmed with Trimmomatic [18] and aligned to the *M. tuberculosis* H37Rv reference
135 genome (GenBank NC000962.3) with BWA [19], SMALT [20] and Novoalign (Novocraft).
136 Genomic variants (single nucleotide variants and insertions and deletions) identified in all

three alignments with SAMTools [21] and the Genome Analysis Toolkit [22] were considered as high confidence variants. Pairwise comparison of the variants identified in progenitor isolates and their corresponding mutants were used to identify unique variants gained or lost during drug exposure and mutant selection. Raw sequence data were also analysed using TB-profiler (version 3.1.12) to infer drug susceptibility profiles and to identify strain lineage [23, 24]. The WGS data were deposited to the European Nucleotide Archive under accession number: PRJEB55505.

Determination of growth rates

The growth rates were determined using the BACTEC MGIT960 platform as previously described [25]. Briefly, cell suspensions with a turbidity equivalent to a McFarland 0.5 standard were created using actively growing cultures (day 21-28) of baseline and mutant strains from Middlebrook 7H10 plates containing OADC. Five hundred microliters of a 1:500 dilution of the cell suspension was used to inoculate MGIT tubes. The growth rate was determined as the time taken for cultures to grow from 5000-10 000 GUs (replicates n=3). A 2-way ANOVA was performed to determine whether there was any statistical difference between the baseline and mutant growth rates.

Results

Each of the selected clinical isolates was genetically characterized by WGS to confirm their lineage classification as well as their genetic drug susceptibility (gDST) pattern (see Tables S1 and S2). WGS confirmed the presence of a single strain in each of the clinical isolates. WGS analysis metrics showed a median depth of coverage of 35× and no evidence of contamination (based on the high percentage of mapped reads, Table S2).

WGS of the progenitor strains revealed that the M2 strain exhibited additional resistance to pyrazinamide, streptomycin and ethambutol; the M3 strain had additional pyrazinamide resistance and the M1 strain had additional streptomycin resistance (Table S1).

Induction of bedaquiline resistance

Bedaquiline-resistant mutants were created by serial passage of H37Rv and each clinical strain on increasing concentrations of each drug (Table S3). Following five passages on bedaquiline-containing media, a range of the highest growth permitting concentrations was observed for the respective clinician strains and H37Rv (0.25- 4 µg/ml, Table S3). Importantly, all bedaquiline-resistant mutants displayed MICs above the critical concentration (CC) of 1 ug/ml (Table 1). WGS identified variants in *atpE* and/or *Rv0678* which included a combination of non-synonymous substitutions and or indels (Table 1). This suggests that the serial selection process selected populations with either a single variant (S1, R1, M2) or multiple variants conferring bedaquiline resistance (S2, M3, H37Rv) or multiple clones with different variants conferring bedaquiline resistance (M1) (Table 1).

The bedaquiline-resistant culture derived from the S1 strain (*Rv0678* frameshift) showed significantly faster growth rates compared to its progenitor strain ($p < 0.01$) (Table 1, Figure S1). Interestingly, the bedaquiline-resistant culture derived from the H37Rv, S2 and M3 strains (with *atpE* variants) all displayed slower growth rates compared to their progenitor strains. From these, only the bedaquiline-resistant culture from the H37Rv strain displayed a significant difference in the growth rate compared to its progenitor strain ($p < 0.01$). Bedaquiline-resistant culture with only *Rv0678* non-synonymous variants (M1,

M2 and R1) all displayed similar growth rates compared to their progenitor strains (Table 1, Figure S1).

Repeated exposure to bedaquiline also selected for variants outside of *atpE* and *Rv0678*. Briefly, variants were identified in *fadE28* (acyl coA dehydrogenase), *truA* (uracil hydrolase), *glnH* (glutamine-binding lipoprotein), *uvrD1* (ATP-dependent DNA helicase), *Rv2366c* (transmembrane protein), *nuoG* (NADH dehydrogenase), *glpK* (glycerol kinase) and *mmpL5* (transmembrane transport protein) (Table 1). The frequency at which these variants appeared ranged from 59 to 100%. Interestingly, two of the mutants derived from the R1 and M1 strains acquired an identical indel in the *glpK* gene. The *mmpL5* variant (A2773G) was also identified in the bedaquiline-resistant mutant derived from the R1 strain. We also identified a synonymous mutation in the bedaquiline-resistant mutant from the R1 strain in the *Rv2326c* gene (C147T) (Table 1).

Induction of clofazimine resistance

Following four passages on clofazimine-containing media, the highest growth permitting concentrations observed for the seven strains was either 1 µg/ml (S2, M1, M3) or 2 µg/ml (H37Rv, S1, R1, M2) (Table S4). WGS showed that variants in *Rv0678* were responsible for clofazimine-resistance. With the exception of H37Rv, variant frequencies were less than 61% - indicative of heteroresistance (Table 2). All of the clofazimine selected cultures displayed MICs higher than the baseline MICs for clofazimine and above the CC of 1 µg/ml, an indication of resistance (Table 2).

In addition to variants in *Rv0678*, WGS identified non-synonymous single nucleotide variants in *ppsD* (polyketide synthase), *fbiD* (*Rv2983*, conserved hypothetical alanine-rich

protein), *Rv0988* (conserved exported protein), *fbiA* (F420 biosynthesis protein), *glpK* (glycerol kinase), *uvrD1* (ATP-dependent DNA helicase), *Rv2082* (conserved hypothetical protein), *nuoG* (NADH dehydrogenase), *glpK* (glycerol kinase) (Table 2). This analysis also identified synonymous mutations in the clofazimine-selected cultures H37Rv and S1 strains in the *Rv3049c* (C549T) and *Rv3299c* genes (C591T), respectively (Table 2).

The H37Rv clofazimine-resistant culture displayed a significantly slower growth rate compared to the progenitor strain ($p<0.01$), while the clofazimine-resistant cultures M1 and R1 displayed significantly faster growth rates ($p<0.01$) (Table S5 and Figure S1).

Intra-group comparison for bedaquiline and clofazimine mutants

Both bedaquiline- and clofazimine-resistant mutant cultures derived from the M1 strain acquired identical *Rv0678* variants, i.e. T461C (Leu154Pro) demonstrating a cross resistance through this variant. Interestingly, both bedaquiline- and clofazimine-resistant mutants cultures derived from the M1 and M2 strains acquired identical *uvrD1* (T1991C: Met664Thr) and *glpK* variants (573insC) respectively (Tables 1 and 2).

Discussion

Bedaquiline and the repurposed drug clofazimine are now considered core agents for treatment of drug resistant TB. The introduction of standardized DST for analysis of phenotypic resistance to both bedaquiline and clofazimine represents a progressive step towards employing regimens that are effective [2]. While rapid genotypic tests and the use of WGS can be used to detect *Rv0678* variants associated with bedaquiline and clofazimine resistance; the release of the WHO 2021 catalogue of drug-resistance

associated mutations three years after endorsing bedaquiline for treatment of drug-resistant TB revealed the paucity of complementary phenotypic and genotypic data for both bedaquiline and clofazimine resistance [6]. This study adds to the current body of knowledge and the results enrich the information contained in existing catalogues [6, 26-28] and shed light on the complexity of pathways involved in resistance acquisition in *M. tuberculosis*.

We identified a single base insertion at position 573 of the *glpK* gene, which extended the length of the homopolymeric tract (position 566-572) from 7C to 8C. The *glpK* gene encodes glycerol 3-phosphotransferase, which is essential for glycerol uptake and metabolism. This variant was found in mutant cultures derived from both Beijing and LAM backgrounds, confirming the finding from Safi *et al.* that transient tolerance can occur in a wide range of phylogenetic lineages and display the same effect [29]. Furthermore, regardless of the drug used for induction (i.e. bedaquiline or clofazimine), the same strains were affected (R1 and M1). Interestingly, despite all the strains being placed under the same experimental conditions, only these two acquired *glpK* frameshift variants. It is unclear if this is due to the genetic background of these specific strains or the labile nature of *glpK* variants. While homopolymers are problematic and error prone in sequencing, the frequency for these variants was >95% for both mutants and the presence of these variants have also been confirmed by other studies [30].

A similar phenomenon was observed with both clofazimine- and bedaquiline-resistant cultures obtained from the M2 strain, i.e. the acquisition of identical *uvrD1* variants (T1991C (Met664Thr)). *UvrD1* encodes for a DNA helicase which unwinds G-quadruplex DNA secondary structures (in an ATP dependent manner) to maintain genome integrity.

While this gene is not essential for mycobacteria survival (unlike *uvrD2*), it has also been shown to be involved in pathogenesis and persistence [31]. *UvrD1* deletion mutants have been found to be hypersusceptible to certain reactive oxygen intermediates and reactive nitrogen intermediates [31]. Although the reduction of clofazimine leads to the formation of reactive oxygen species (ROS) [11], it is unclear if the variant identified in *uvrD1* would ameliorate the effect of ROS in the bacteria. Additionally, ROS production was not found to be increased by bedaquiline in other studies [32].

The progenitor M1 strain harboured a low-frequency (<30%) *nuoG* variant. Following exposure to either bedaquiline or clofazimine resulted in a mutant population with a high frequency *nuoG* variant, particularly for the clofazimine mutant population (0.95 versus 0.59). *nuoG* is a virulence gene belonging to the *nuo*-operon, containing 14 genes, which code for NADH dehydrogenase Type I (Ndh-1) [33]. The original hypothesis was that clofazimine requires activation *via* Ndh-2 [11], with limited data pointing to Ndh-1 in *M. tuberculosis* [34-36]. Ndh-2 is the primary dehydrogenase used by *M. tuberculosis* and is essential for the survival of the bacteria, while Ndh-1 is a proton-pumping dehydrogenase, found to be non-essential for bacterial growth [35]. Both of these enzymes appear to play active roles in maintaining NADH homeostasis [35]. Interestingly, *M. leprae* only has the *nuoN* pseudogene and the entire *nuo*-operon is deleted in this mycobacterial species [37], which may explain the efficacy of clofazimine as a leprosy drug having only one NADH dehydrogenase to target. Alternatively, the role of *nuoG* in these bedaquiline and clofazimine mutants may be explained by interference with the proton motive force (through bedaquiline affecting ATP synthase or clofazimine affecting Ndh-2), which may

impact the expression of certain genes and the preferred use of different enzymes (proton-pumping Ndh-1 vs non-proton pumping Ndh-2) accordingly [34].

Rv0678 variants were the most common variants in both bedaquiline- and clofazimine-resistant mutants derived from all seven strains. These variants were never observed on their own, but rather in combination with other *Rv0678* variants, other *atpE* variants or other variants in non-canonical pathways. When multiple low frequency *Rv0678* variants occurred, we analysed the alignments and these appeared to belong to distinct sub-populations within the sequenced mutant population. In the case of high frequency *Rv0678* and *atpE* variants, we previously showed that *atpE* related resistance is likely the final step in high-level bedaquiline resistance [38], which could explain the lack of occurrence of independent *atpE* variants. However, an additional factor to consider is the genetic background of the strain, which appears to influence the variant acquired as our previous study showed that fully susceptible or mono-resistant *M. tuberculosis* reference strains used for bedaquiline mutant generation display independent *atpE* variants [13]. The acquisition of an *atpE* variant could also be associated with a concurrent loss of fitness. The slower growth rate in the bedaquiline-resistant mutant cultures which acquired *atpE variants* (i.e. from the H37Rv, S2 and M3 strains) could be evidence for this, however, a statistically significant difference was not shown for the latter two strains. The acquisition of variants other than *atpE* and *Rv0678* could be an indication of the complex number of pathways associated with resistance to these two drugs.

Cell wall biosynthesis pathways have been previously postulated to accommodate shifts due to resistance [16]. In this study, we show that multiple genes are involved, in particular those used to maintain homeostasis along the cell membrane. However, other than the

genes mentioned above, all variants detected in other genes were single events and did not overlap, neither between strains nor between drugs. Of concern however, were the *fbiD* and *fbiA* variants identified in two distinct clofazimine-resistant mutants derived from the H37Rv and R1 strains. FbiD (*Rv2983*) is a phosphoenolpyruvate (PEP) guanylyltransferase that synthesizes the phosphoenolpyruvyl moiety, which is subsequently transferred to F0 by FbiA [39]. *fbiA* encodes a 2-phospho-L-lactate transferase, which transfers the lactyl phosphate moiety of lactyl-2-diphospho-5-guanosine to 7,8-didemethyl-8-hydroxy-5-deazariboflavin in the F420 biosynthesis pathway [42, 43]. While *fbiA* is not essential for *M. tuberculosis in vitro* growth, it was found that variants in the *fbiA* gene alter the production of F420 [44]. Both *fbiA* variants identified in this study (T862C and 283delTG) appear in close proximity to those previously reported in delamanid- and pretomanid-resistant isolates [44]. The enzymes involved in the F420 biosynthesis pathway are well known to be involved in resistance to nitroimidazoles (delamanid and pretomanid) [45]. Additionally, *fbiD* mutants have been found to be cross-resistant to both pretomanid and delamanid [40]. While it has been previously demonstrated that an F420 deficient pretomanid-resistant *fbiD* mutant is hypersusceptible to clofazimine [40], to our knowledge, this is the first study, which demonstrates the acquisition of a variant in fbiD following exposure to clofazimine. Another study by Waller et al, [41], has shown the selection of both *fbiA* and *fbiC* variants following clofazimine exposure and confirmed the role of genetic variants in the F420 biosynthesis pathway (besides *ddn*) with low-level clofazimine cross-resistance. Currently, with pretomanid and bedaquiline being used together in the BPaL regimen, investigating this association is critical to ensure protection of the TB drug arsenal.

316 Finally, the *mmpL5* gene encodes for the protein involved in the mmpS5-mmpL5 efflux
317 pump, which is regulated by *mmpR* (encoded by *Rv0678*) [3]. The *mmpL5* variant
318 acquired by the R1 strain in the bedaquiline-resistant mutant was not observed in the
319 progenitor strain and is therefore unlikely to be phylogenetically relevant. However, this
320 variant co-occurs with an *Rv0678* variant (T416G) and the WHO mutant catalog has
321 classified an *Rv0678* variant at this nucleotide position (T416C) as having uncertain
322 significance as it was only previously identified in two BDQ sensitive strains [6]. This could
323 mean that the mmpL5 variant could be responsible for the observed MIC increase or
324 resistance or could play a role in compensation (if the *Rv0678* variant is key to the
325 acquired resistance observed). *et al.*

326 The use of a small sample size is a key limitation in this study as the vast diversity of the
327 non-canonical variants identified also now require further confirmation. The presence of
328 overlapping variants between bedaquiline- and clofazimine- resistant mutant cultures
329 could be as a result of the strain becoming culture adapted or an undetected underlying
330 population (particularly in the case of the underlying *nuoG* population) being specifically
331 selected due to serial drug exposure. Furthermore, the use of mutant populations rather
332 than single clones (as evidenced by variant frequencies from WGS data) could impact
333 the findings from the growth rate studies. However, purifying to a single clone level would
334 not show the diversity of mutations that can arise following induction on drug-containing
335 media. Future research should focus on phenotypic drug susceptibility testing for
336 delamanid and pretomanid for the *fbiA* and *fbiD* mutants. Additionally, WGS after the final
337 passage could be compared with WGS analysis following each passage to compare the
338 accumulation of resistance. Finally, comprehensive genomic and possibly transcriptomic

analysis, within these resistant *M. tuberculosis* strains could be performed to further elucidate mechanisms of action for these drugs.

In conclusion, we show the potential of *in vitro* resistance induction to both bedaquiline and clofazimine without apparent loss of fitness (especially in the presence of *Rv0678* variants), which is highly concerning. While the limited number of isolates in this study may provide only a cursory glance, it is plausible that the genetic background may influence the type of variant selected for and the degree of impact on associated pathways, as seen in larger studies focused on *Rv0678* related resistance [46]. There appears to be a putative link between transient tolerance (*glpK*), *nuoG* and *uvrD1* genes and bedaquiline and clofazimine resistance. The unique genes implicated in the resistance acquisition for bedaquiline and clofazimine suggests that *M. tuberculosis* uses a complex involvement of pathways required to maintain fitness to accommodate the shift from a susceptible to resistant genotype.

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Figure 1: Experimental workflow for progenitor sample set characterization, mutant generation and phenotypic and genotypic mutant characterization. Abbreviations: NICD: National Institute for Communicable Diseases, DST: drug susceptibility testing, MIC: minimal inhibitory concentration, MGIT: mycobacteria growth indicator tube

Table 1: Comparison of baseline phenotypic data for progenitor strains compared to phenotypic (MIC and length of lag phase and growth rates) and genotypic data for bedaquiline-resistant mutants.

Strain	Bedaquiline MIC in µg/ml (MGIT)		Variants acquired						Average growth rate (hrs)	
	Base line	Mutant	*Rv0678*		*atpE*		Other		Base line	Mutant
			NT (AA)	Freq.	NT (AA)	Freq.	Gene: NT (AA)	Freq.		
H37Rv	≤0.125	>8	T2C (Val1Ala)	0.46	A83G (Asp28Gly)	0.65	*fadE28:* C578T (Ala193Val)	1.00	19	41 (*)
					G183C (Gly61Asp)	1.00	*ponA2:* G1614C (syn)	0.18		
S1	0.5	8	90_91insA	1.00	-		*truA:* 8insG	0.76	34	24 (*)
S2	0.5	>8	G74A (Gly25Asp)	1.00	C188T (Ala63Val)	1.00	-	-	23	31
R1	≤0.125	8	T416G (Met139Arg)	0.97	-	-	*mmpL5:* A2773G (Met925Val)	0.96	35	36
							glnH: G712C (Ala238Pro)	1.00		
							pks8: C505G (Leu169Val)	0.93		
							glpK: 573insC	1.00		
							Rv2326c: C147T (syn)	1.00		
M1	1	4	G203A (Ser68Asn)	0.25	-	-	*nuoG:* G1246C (Ala416Pro)[a]	0.59	31	28
			T374C (Leu125Pro)	0.23			*glpK* 573insC	0.96		
			431delAT	0.18						
M2	≤0.125	4	T461C (Leu154Pro)	1.00	-	-	*uvrD1:* T1991C (Met664Thr)	1.00	28	23
M3	1	8	66_67insT	1.00	G183T (Glu61Asp)	1.00	114 bp insertion between *Rv3680* and *whiB4*	1.00	46	76

[a] *nuoG* variant present in progenitor at lower frequency.
Synonymous variants indicated in brackets (syn).
NT indicates the nucleotide change and AA indicates the amino acid change.
Freq. indicates the allele frequency for the variant.
Statistically significant differences between mutant and baseline for lag phase and growth rates (hours, hrs) are indicated by *,**,***, which represent p-values of <0.05, <0.01, <0.001 respectively.

Table 2: Comparison of baseline phenotypic data for progenitor strains compared to phenotypic (MIC and length of lag phase and growth rates) and genotypic data for clofazimine-resistant mutants.

	CFZ MIC in µg/ml (MGIT)		Variant acquired				Average growth rate (hrs)	
Strain	**Base line**	**Mutant**	***Rv0678***		**Other**		**Base line**	**Mutant**
			NT (AA)	**Freq.**	**Gene: NT (AA)**	**Freq.**		
H37Rv	0.125	4	211insC	1.00	*ppsD:* T3518C (Leu1173Pro);	1.00	19	35 (**)
					fbiD: del555GCT	0.51		
					Rv3049c: C549T (syn)	1.00		
S1	0.5	4	192_193delG	0.61	*atsB*: C591T (syn)	0.77	34	42
			T167C (Leu56Pro)	0.17	*mutT3:* T155A (L52Q)	0.2		
					fadE18: G608T (R203D)	0.22		
					fadE18: A607C (R203D)	0.22		
S2	0.5	2	192_193delG	0.49	-		23	36
			A208G (Asn70Asp)	0.23				
			G404C (Arg135Pro)	0.24				
R1	0.5	4	A65C (Gln22Pro)	0.48	*Rv0988:* C493G (Arg165Gly)	0.57	35	84
					fbiA: 283delTG	0.39		
			166_177del ACTGGCGACGGCG	0.40	*fbiA:* T862C (Trp288Arg)	0.31		
					glpK: 573insC	1.00		
M1	0.5	4	T461C (Leu154Pro)	0.31	*nuoG:* G1246C (Ala416Pro)[a]	0.95	31	33
			274insA	0.2	*glpK:* 573insC	1.00		
M2	0.5	2	C251T (Ala84Val)	0.51	*uvrD1:* T1991C (Met664Thr)	0.79	28	23 (*)
			G404C (Arg135Pro)	0.09	*Rv2082:* G2083A (Ala695Thr)	0.18		
			465insC	0.21				
M3	0.5	4	T128C (Leu43Pro)	0.17	-	-	46	49
			T131C (Leu44Pro)	0.16				
			G215A (Arg72Gln)	0.2				

[a] *nuoG* variant present in progenitor at lower frequency.
Synonymous variants indicated in brackets (syn).
NT indicates the nucleotide change and AA indicates the amino acid change.
Freq. indicates the allele frequency for the variant.
Statistically significant differences between mutant and baseline for lag phase and growth rates (hours, hrs) are indicated by *,**,***, which represent p-values of <0.05, <0.01, <0.001 respectively.