In Vitro Study of Stepwise Acquisition of rv0678 and atpE Mutations Conferring Bedaquiline Resistance

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Running title: Acquired *atpE* bedaquiline resistance

Abstract

Bedaquiline resistance within *Mycobacterium tuberculosis* may arise through efflux- (*rv0678*) or target-based (*atpE*) pathway mutations. *M. tuberculosis* mutant populations from each of five sequential steps in a passaging approach, using a pyrazinamide-resistant ATCC strain, were subjected to MIC determinations and whole-genome sequencing. Exposure to increasing bedaquiline concentrations resulted in increasing phenotypic resistance (up to >2 µg/mL) through minimal inhibitory concentration determination on solid media (Middlebrook 7H10). *Rv0678* mutations were dynamic while *atpE* mutations were fixed, once occurring. We present a hypothesis for *in vitro* emergence of bedaquiline resistance: *rv0678* mutations may be the first transient step in low-level resistance acquisition followed by high-level resistance due to fixed *atpE* mutations.

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Key words: bedaquiline, stepwise acquisition, *rv0678*, *atpE*, in vitro model

Introduction

Mycobacterium tuberculosis is capable of acquiring resistance to multiple drugs due to intrinsic, efflux and target-based pathways; key characteristics that undermine treatment success and affect morbidity and mortality.

The inclusion of bedaquiline, a novel drug, into regimens for drug-resistant tuberculosis (DR-TB) is an important step forward towards improving treatment outcomes. The early success of bedaquiline, however, is threatened by potential emergence of resistance due to poor adherence to TB treatment, relatively weak background regimens and the long half-life of the drug (1, 2). Interest around bedaquiline has spiked with the release of the WHO rapid communication suggesting that bedaquiline employment be prioritised and used to replace injectable drugs (3). However, bedaquiline treatment failure from clinical cases has already been reported (4, 5), with the majority of resistance due to mutations in the efflux-associated pathway. These mutations occur in the *rv0678* gene encoding the *MmpR* repressor protein, which influences transcription of the *MmpL5-MmpS5* efflux pump proteins (6). Alternatively, mutations in the *atpE* gene, the primary drug target for bedaquiline, have only been identified in two clinical isolates (7); only one of these was classified as phenotypically resistant according to the EUCAST susceptible breakpoint ($\leq$0.25 µg/mL).

To clarify the potential pathway through which *M. tuberculosis* bacteria acquire and evolve resistance to bedaquiline, we present a hypothesis based on in vitro generation of mutant populations.

Materials and Methods

A pyrazinamide mono-resistant reference strain, ATCC35828 (*pncA*: Gly132Ser mutation), which we previously observed displaying higher baseline bedaquiline MIC values and rapidly accumulating resistance to bedaquiline compared to a fully-susceptible and other mono-resistant strains (8) was exposed to increasing concentrations of bedaquiline during a serial passage approach to obtain five sequential *M. tuberculosis* mutant populations as previously described (8). In brief, a McFarland 1.0 cell suspension of a 21-28 day old culture was used to inoculate (100 µL) four 7H10 plates. These were either drug-free (growth control) or containing bedaquiline at 0.5×, 1× and 2× the proposed critical concentration (0.25 µg/mL). Cultures were grown at 37^{o}C for 21-28 days or until sufficient growth was observed. Following this passage, confluent growth was scraped from the plate with the highest drug concentration permitting growth (Figure 1). This growth was used for the creation of a new cell suspension for minimal inhibitory concentration (MIC) determinations (below) and the following passage. Again, four plates were inoculated; a drug-free control; a plate with the growth-permitting drug concentration (same as which growth was scraped from) as well as plates with 2- and 4-fold higher drug. This process was repeated until a total of five of these passages was completed.

MIC values were determined using Middlebrook 7H10 solid agar (Sigma-Aldrich) as previously described (9). In brief, cell suspensions (McFarland 1.0) were prepared from actively growing cultures for baseline and mutant strains. A ten-fold dilution of this suspension was used to inoculate (100 µL) a series of bedaquiline-containing solid agar plates (Range: 0.004 - 2 µg/mL). Three further dilutions were used to inoculate drug-free plates; to control inoculum size and serve as positive

controls for growth. Plates were incubated at 37ºC for 21 days. The MIC was determined as the lowest concentration within the series with 100% visible growth inhibition.

Genomic DNA extraction was performed on the NucliSENS easyMAG platform (BioMérieux, Marcy-l'Étoile, France) using 500µl heat-killed and bead-beaten cultured isolate, with the instrument's generic protocol to obtain a final eluate (25µl) of purified nucleic acids. Preparation of paired-end libraries was performed using the Nextera XT DNA library kit (Illumina, San Diego, CA, USA), following manufacturer's protocol for tagmentation, size selection and a modified library normalization step (10). WGS was carried out using the Illumina MiSeq with the 2× 300bp V3 cartridge.

Identification of *rv0678* and *atpE* mutations was performed using the CLC Genomics workbench (version 10) and by mapping to an edited, annotated, reference *M. tuberculosis* H37Rv genome (Genebank NC000962.3). The following parameters were set to identify single nucleotide polymorphisms (SNPs) or insertions/deletions (indels): forward/reverse balance >0.015, minimum coverage depth of 5×, frequency of ≥30%, length and similarity fractions of 0.8 each and a PHRED score of ≥Q20 (≥99% accuracy) at both variant positions and nucleotides within a 5bp radius. Additionally, using the same stringent quality measures, we reduced the frequency to 1% to observe fluctuation in *rv0678* and *atpE* mutations.

Results

The baseline solid bedaquiline MIC concentration was 0.06 µg/mL, classified as sensitive, for the ATCC reference strain. Phenotypic resistance, was observed after the first passage, with solid agar MIC values of 1 µg/mL, which increased to 2 and then >2 µg/mL from the second passage onwards. Similarly, the highest drug concentration permitting growth on solid media was observed to increase from the first to the fourth passage from 0.5 to 4 µg/mL (Figure 1).

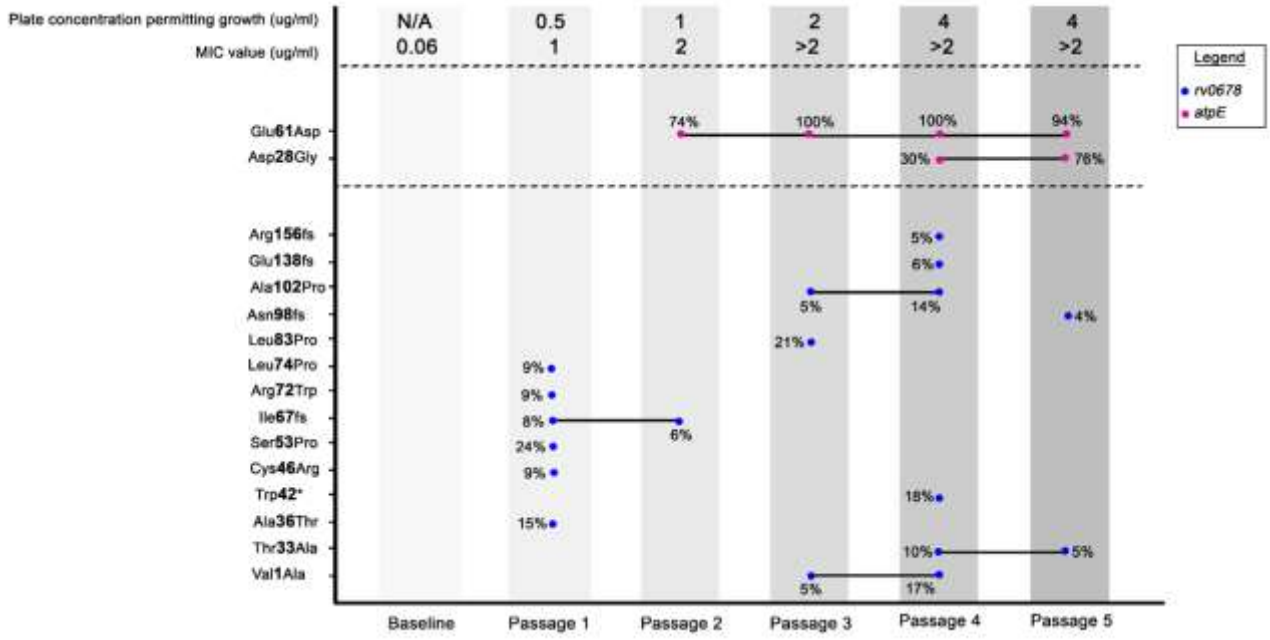

Figure One: Graph depicting the *rv0678* and *atpE* mutations accumulated over five passages and the frequencies at which they appear. Mutations appearing over more than a single passage are connected by a line. (MIC determined using solid media for baseline and mutant populations, EUCAST breakpoint of 0.25 µg/mL used)

In total, fourteen distinct *rv0678* mutations but only two *atpE* mutations were identified in five passages, Figure 1 details the passage at which they appeared. Four of the fourteen *rv0678* mutations (Ile67fs, Val1Ala, Ala102Pro, Thr33Ala) were identified in two passages, but at low frequency. The total number of *rv0678* mutations

in each population fluctuated (Figure 1: 6→1→3→6→1) over passaging. On the other hand, a single *atpE* mutation, Glu61Asp, was maintained for four passages, while the Asp28Gly *atpE* mutation was maintained for the final two passages. In the final passage, certain mutants possessed two *atpE* mutations concurrently with rv0678 mutations. No *atpE* mutations were detected in the first passage.

Discussion

We present an experimental hypothesis of the underlying mutation dynamics associated with in vitro bedaquiline resistance acquisition in *M. tuberculosis*. Five sequential *M. tuberculosis* populations obtained through serial passaging were investigated using WGS. Although a variable profile of resistance-associated mutations was identified in each passage step, the phenotypic profile was consistently resistant progressing from low to high MIC values.

Rv0678 mutations were identified in the first passage and the number and frequency of *rv0678* mutations decrease on appearance of the Glu61Asp *atpE* mutation. Similarly, when the Asp28Gly *atpE* mutation appears at a high frequency (76%), the number and frequency of *rv0678* mutations decrease. As such, certain *rv0678* mutations may appear as precursors to high-level resistance caused by *atpE* mutations (11). Additionally, using a catalogue of *rv0678* collated from data (12), we found that the majority of the *rv0678* mutations (or positions mutated) reported in this publication (9/14) have been associated with low-level resistance either clinically or in vitro. We find it unlikely that an organism that is already low-level resistant would proceed to procure further mutations unless high-level resistance could be ensured.

In mutant populations with only *rv0678* mutations, the solid agar MIC value was 1 µg/mL and in populations with *atpE* mutations, the MIC was 2 to >2 µg/mL. Intermediate concentrations (1-2 µg/mL) could possibly select for both mutation types. Lower MIC values appear to be associated with *rv0678* mutations, whereas higher MIC values with *atpE* mutations. From literature we find that spontaneous bedaquiline resistant mutants possessing only *atpE* mutations and either *rv0678* or *atpE* could be selected at a 1 (8) or 0.9 µg/mL (13) concentrations, respectively. However, both these studies are performed using a spontaneous approach rather than serial passaging.

As confluent growth or an entire mutant population was scraped from plates, frequency of mutations observed may be representative of their occurrence in the population and cannot be assigned to a single colony. Literature shows that a single *atpE* mutation is sufficient to result in bedaquiline resistance (13) and the same holds true for *rv0678* mutations (6). While it seems unlikely that multiple *rv0678* or *atpE* mutations would readily occur in a single organism, in this study, the Glu61Asp *atpE* mutation occurs at 100% alongside low frequency *rv0678* mutations, leading us to assume that every organism sequenced within this population displayed this *atpE* mutation and some concurrent *rv0678* mutations. Using an *in vitro* data set comprised of clinical strains, dual mutants were observed, though this work is yet to be published (Ismail N – personal communication). We also saw dual mutants in isoniazid-, rifampicin- and kanamycin-mono resistant strains (8). In a study by Zimenkov *et al.*, a clinical isolate from an XDR patient exhibited both *atpE* and *rv0678* mutations (7). Certain *rv0678* mutations, possibly those in passages three to five, could be responsible for increased efflux pump expression due to the high drug concentration faced by the organism. Conversely, these same *rv0678* mutations could be responsible for decreased efflux pump expression as the organism has acquired *atpE*

mutations to withstand the increased drug pressure. Further investigation of the association of different *rv0678* mutations to the level of resistance is necessary to understand this phenomenon.

In this study, the Asp28Gly *atpE* mutation became apparent after the mutation at position 61 had occurred, initially at a 30% frequency, followed by an increase to 76% in the next passage. We postulate the appearance of this mutation could be a compensatory mutation. It is possible that if a sixth passage were investigated, the frequency could increase further as it did with the Glu61Asp mutation. While both mutations have been previously described by Segala *et al.* (14) to be associated with bedaquiline resistance (solid MIC: 0.5-1 µg/mL), they also show that mutations at position 28 exhibit greater diversity and this residue is positioned at the base of the *atpE* binding pocket while Glu61 is positioned at the edge of the binding pocket. Zimenkov *et al.* show that the Asp28Gly mutation found in clinical isolates do not confer resistance (7). While the Asp28Gly *atpE* mutation in vivo may play a compensatory role to increase fitness of the organism rather than be associated with resistance, further experiments investigating fitness as well as investigation of additional strains possessing this mutation may prove useful.

The hypothesis presented in this study, derived both from literature as well as observations in vitro, is depicted graphically in Figure 2. Here, we see that a bedaquiline resistant population may arise due to the presence of *rv0678* mutations, which are either induced from bedaquiline exposure or from other drugs inducing *rv0678* mutations (15). Other studies have shown that the *M. tuberculosis* populations that appear within lesions exhibit heterogeneity and may acquire resistance through acquisition of dynamic mutations (16, 17). Here, through the investigation of a *M.*

tuberculosis in vitro population, we observe a population with transient *rv0678* mutations with a lower MIC value than the populations possessing fixed *atpE* mutations (Figure 1). These results could explain why the majority of bedaquiline resistant clinical cases possess *rv0678* mutations as opposed to *atpE* mutations. The effective concentration of bedaquiline inside granulomas is as yet unknown and poor penetration could possibly explain the low number of *atpE* mutations observed clinically (Figure 2) (18). Additionally, it is not possible to determine whether low-level or intermediate resistance caused by dynamic *rv0678* mutations could be overcome by treating with higher concentrations of bedaquiline as increased doses of bedaquiline impact patient cardiac health.

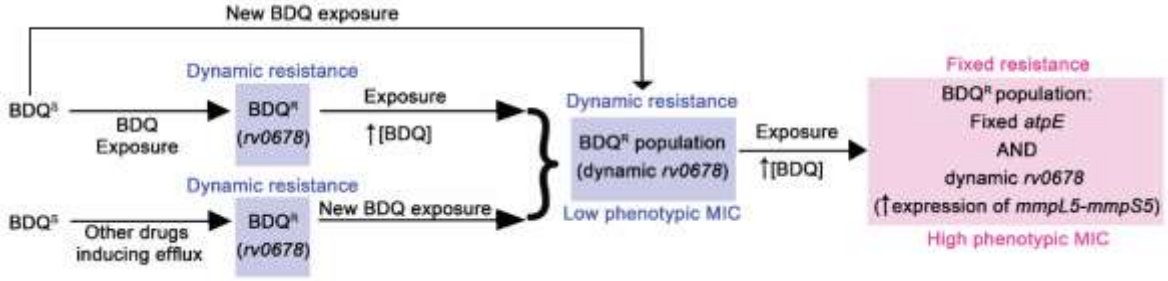

Figure Two: A hypothesis for acquisition of bedaquiline resistance due to drug exposure. A bedaquiline resistant population arises due to the presence of *rv0678* mutations, either from other drugs influencing the efflux pathway (*rv0678*) or via bedaquiline exposure in suboptimal treatment scenarios. With increased drug pressure or new bedaquiline exposure, a resultant population with dynamic *rv0678* mutations may be observed and these mutations may fluctuate within the population over time. Application of further pressure could result in a population with fixed *atpE* mutations, which may arise and are maintained over time. This population may concurrently comprise of bacteria with *rv0678* mutations, which are responsible for the increased expression of *mmpL5-mmpS5* efflux pumps. While both populations are bedaquiline resistant (BDQ^R), the population with *atpE* mutations possesses higher MIC values than the population with *rv0678* mutations only. (BDQ=bedaquiline)

Although more than one of these observations may be plausible, there are limitations that must be taken into account. For extrapolation to an in vivo scenario, a key limitation is the exposure to only bedaquiline in our experimental set-up as compared to combination therapy in clinical practice. While suboptimal DR-TB treatment scenarios may be responsible for the appearance of *rv0678* mutations in bedaquiline resistant clinical isolates, the presence of other supporting drugs may prevent the acquisition of high-level resistance in the form of *atpE* mutations. Other possible avenues to pursue to understand the effects of drug exposure would be to expose a mutant population to a single concentration of bedaquiline over five passages. This could clarify whether *atpE* mutations appear as a result of prolonged drug exposure or driven through exposure to increasing drug concentration. Additionally, given the long half-life of bedaquiline in vivo future experiments could be performed to observe the effect of exposure to decreasing concentrations of drug. While functional genomics studies to investigate the role of the *atpE* mutations in hotspot regions would be useful, an in-depth study of these has been previously performed by Segala *et al.* (14). For *rv0678* mutations, previously described attempts to clone *rv0678* variants into integrative vectors showed partial but not complete resistance as the wild type *rv0678* gene is still expressed (6). Dynamic changes from *rv0678* to *atpE* mutations could also be further investigated through the use of a single clone as opposed to a mutant population.

We provide a hypothesis for the evolution of bedaquiline resistance through the acquisition of *rv0678* and *atpE* mutations. These findings show that *rv0678* mutations result in low-level bedaquiline resistance. *Rv0678* mutations have been identified in bedaquiline resistant clinical isolates, clofazimine-resistant clinical isolates and in clinical isolates without prior exposure to either drug, overview in (18). Thus, the

finding that certain *rv0678* mutations may behave as precursors for the appearance of *atpE* mutations is a cause for concern. While a number of rv0678 mutations have been identified that are not associated with bedaquiline resistance (19), it would be useful to have a comprehensive catalogue of *rv0678* mutations with associated MICs as well as those which are linked to step-wise acquisition of *atpE*-based bedaquiline resistance. As mutations in the *rv0678* gene are scattered throughout the gene, genotypic screening of the entire gene for mutations may prove useful to identify those mutations that may negate bedaquiline usage.

Data availability

Raw sequence data can be found on the NCBI platform under the Bioproject accession number: PRJNA517607.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Singh P, Kumari R, Lal R. 2017. Bedaquiline: Fallible Hope Against Drug Resistant Tuberculosis. Indian J Microbiol 57:371-377.
2. Nguyen TVA, Anthony RM, Banuls AL, Nguyen TVA, Vu DH, Alffenaar JC. 2018. Bedaquiline Resistance: Its Emergence, Mechanism, and Prevention. Clin Infect Dis 66:1625-1630.
3. WHO. 2018. Rapid communication: Key changes to treatment of multidrug- and rifampicin-resistant tuberculosis (MDR/RR-TB)
4. Bloemberg GV, Keller, P. M., Stucki, D., Trauner, A., Borrell, S., Latshang, T., Coscolla, M., Rothe, T., Hömke, R., Ritter, C., Feldmann, J., Schulthess, B., Gagneux, S. and Böttger, E. C. 2015. Acquired Resistance to Bedaquiline and Delamanid in Therapy for Tuberculosis. N Engl J Med 373:1986-1988.
5. Zimenkov DV, Nosova, E. Y., Kulagina, E. V., Antonova, O. V., Arslanbaeva, L. R., Isakova, A. I., Krylova, L. Y., Peretokina, I. V., Makarova, M. V., Safonova, S. G., Borisov, S. E. and Gryadunov, D. A. 2017. Examination of bedaquiline- and linezolid-resistant *Mycobacterium tuberculosis* isolates from the Moscow region. J Antimicrob Chemother 72:1901-1906.
6. Hartkoorn RC, Uplekar S, Cole ST. 2014. Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in Mycobacterium tuberculosis. Antimicrob Agents Chemother 58:2979-81.
7. Zimenkov DV, Nosova EY, Kulagina EV, Antonova OV, Arslanbaeva LR, Isakova AI, Krylova LY, Peretokina IV, Makarova MV, Safonova SG, Borisov SE, Gryadunov DA. 2017. Examination of bedaquiline- and linezolid-resistant Mycobacterium tuberculosis isolates from the Moscow region. J Antimicrob Chemother 72:1901-1906.
8. Ismail N, Omar SV, Ismail NA, Peters RPH. 2018. In vitro approaches for generation of Mycobacterium tuberculosis mutants resistant to bedaquiline, clofazimine or linezolid and identification of associated genetic variants. J Microbiol Methods 153:1-9.
9. Kaniga K, Cirillo DM, Hoffner S, Ismail NA, Kaur D, Lounis N, Metchock B, Pfyffer GE, Venter A. 2016. A Multilaboratory, Multicountry Study To Determine Bedaquiline MIC Quality Control Ranges for Phenotypic Drug Susceptibility Testing. J Clin Microbiol 54:2956-2962.
10. Omar SV, Joseph L, Said HM, Ismail F, Ismail N, Gwala TL, Ismail NA. 2019. Whole genome sequencing for drug resistance determination in Mycobacterium tuberculosis. Afr J Lab Med 8:a801.
11. Andries K, Villellas C, Coeck N, Thys K, Gevers T, Vranckx L, Lounis N, de Jong BC, Koul A. 2014. Acquired resistance of Mycobacterium tuberculosis to bedaquiline. PLoS One 9:e102135.

12. Ismail N, Omar SV, Ismail NA, Peters RPH. 2018. Collated data of mutation frequencies and associated genetic variants of bedaquiline, clofazimine and linezolid resistance in Mycobacterium tuberculosis. Data in brief 20:1975-1983.
13. Huitric E, Verhasselt P, Koul A, Andries K, Hoffner S, Andersson DI. 2010. Rates and mechanisms of resistance development in Mycobacterium tuberculosis to a novel diarylquinoline ATP synthase inhibitor. Antimicrob Agents Chemother 54:1022-8.
14. Segala E, Sougakoff W, Nevejans-Chauffour A, Jarlier V, Petrella S. 2012. New mutations in the mycobacterial ATP synthase: new insights into the binding of the diarylquinoline TMC207 to the ATP synthase C-ring structure. Antimicrob Agents Chemother 56:2326-34.
15. Villellas C, Coeck N, Meehan CJ, Lounis N, de Jong B, Rigouts L, Andries K. 2017. Unexpected high prevalence of resistance-associated Rv0678 variants in MDR-TB patients without documented prior use of clofazimine or bedaquiline. J Antimicrob Chemother 72:684-690.
16. Liu Q, Via LE, Luo T, Liang L, Liu X, Wu S, Shen Q, Wei W, Ruan X, Yuan X, Zhang G, Barry CE, 3rd, Gao Q. 2015. Within patient microevolution of Mycobacterium tuberculosis correlates with heterogeneous responses to treatment. Sci Rep 5:17507.
17. Sun G, Luo T, Yang C, Dong X, Li J, Zhu Y, Zheng H, Tian W, Wang S, Barry CE, 3rd, Mei J, Gao Q. 2012. Dynamic population changes in Mycobacterium tuberculosis during acquisition and fixation of drug resistance in patients. J Infect Dis 206:1724-33.
18. Sarathy JP, Zuccotto F, Hsinpin H, Sandberg L, Via LE, Marriner GA, Masquelin T, Wyatt P, Ray P, Dartois V. 2016. Prediction of Drug Penetration in Tuberculosis Lesions. ACS Infect Dis 2:552-63.
19. Villellas C, Coeck, N., Meehan, C. J.,Lounis, N., de Jong, B.,Rigouts, L. and Andries, K. 2017. Unexpected high prevalence of resistance-associated Rv0678 variants in MDR-TB patients without documented prior use of clofazimine or bedaquiline. J Antimicrob Chemother 72:684-690.