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Identification of novel mutations associated with clofazimine resistance in *Mycobacterium tuberculosis*

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Objectives: Although clofazimine has been traditionally used to treat leprosy, there is recent interest in using clofazimine for the treatment of MDR-TB and drug-susceptible TB. However, the mechanisms of resistance to clofazimine are poorly understood. Here, we investigated the molecular basis of clofazimine resistance using resistant mutants isolated *in vitro*.

Methods: We isolated 96 mutants of *Mycobacterium tuberculosis* resistant to clofazimine and performed WGS and Sanger sequencing to identify possible mutations associated with clofazimine resistance.

Results: We found that 97% (93/96) of clofazimine-resistant mutants had a mutation in *rv0678* encoding a transcription repressor for efflux pump MmpL5. Two mutational hot spots at nucleotide positions 193 and 466 in *rv0678* accounted for 43.8% (42/96) and 11.5% (11/96) of the mutations, respectively. The previously reported A202G mutation (S68G) in *rv0678* occurred less frequently, in 5 of 96 mutants. The remaining 34 mutations were scattered along the entire *rv0678* gene. We discovered two new genes (*rv1979c* and *rv2535c*) associated with clofazimine resistance in mutants without *rv0678* mutations.

Conclusions: Mutations in *rv0678* are a major mechanism of clofazimine resistance. Our findings provide useful information for the design of new molecular tests for rapid detection of clofazimine resistance. Further studies are needed to address the role of *rv1979c* and *rv2535c* in clofazimine resistance and mechanisms of action.

Introduction

Clofazimine is a riminophenazine drug that was developed as an anti-TB agent in the 1950s,[1] but is commonly used to treat leprosy.[2] Although it has good activity against *Mycobacterium tuberculosis in vitro*, it was not used in the treatment of pulmonary TB.[3] Due to increasing emergence of MDR-TB, there has recently been interest in the use of clofazimine (a Group 5 agent) to treat MDR-TB[4,5] and its use might shorten MDR-TB treatment to 9 months.[6] A recent study provides further support for clofazimine being involved in shortening the treatment of drug-susceptible TB in the mouse model.[7]

Although clofazimine was discovered in the 1950s, its exact mechanism of action has remained obscure. It appears to have multiple effects on the organism, such as interfering with redox cycling,[1] causing membrane destabilization and dysfunction[8] and the production of reactive oxygen species.[9] The mechanism of resistance to clofazimine in *M. tuberculosis* was unknown. Recently, mutations in the *rv0678* gene, which encodes a transcription repressor of efflux pump MmpS5-MmpL5, causing overexpression of the efflux pump, were found to result in cross-resistance to both clofazimine and bedaquiline (TMC207).[10,11] Clinical strains isolated as resistant to bedaquiline during clinical trials of this drug have recently been shown to have *rv0678* mutations that cause cross-resistance to clofazimine.[11,12] However, no clinical isolates resistant to clofazimine have been reported and only a few mutants isolated *in vitro* have been analysed so far.[10,11] To better understand the mechanism of clofazimine resistance and to develop more rapid molecular tests for detection of resistance, we characterized 96 clofazimine-resistant mutants isolated *in vitro* from *M. tuberculosis* H37Rv and found many new mutations in the *rv0678* gene that had not been previously reported. In addition, we identified two new genes (*rv1979c* and *rv2535c*) that are associated with clofazimine resistance in *M. tuberculosis*.

Materials and methods

Mutant isolation

One-month-old *M. tuberculosis* H37Rv independent cultures grown in 7H9 liquid medium were plated on 7H11 plates containing 0.25, 0.5 or 1 mg/L clofazimine. After incubation at 37°C for 4 weeks, the mutant colonies were picked to confirm the drug resistance phenotype by transferring the mutants onto new plates containing different concentrations of clofazimine (0.25, 0.5, 1 or 2 mg/L).

Drug susceptibility testing

Drug susceptibility testing of the clofazimine-resistant mutants was performed on 7H11 agar plates containing 0.25, 0.5, 1 or 2 mg/L clofazimine as described previously.[13] WT *M. tuberculosis* H37Rv was included as a drug-susceptible control. The clofazimine-susceptible strain H37Rv did not grow on clofazimine-containing plates and only mutants that were consistently resistant to clofazimine were further analysed by PCR and DNA sequencing, as described below.

WGS

Genomic DNA from *M. tuberculosis* H37Rv mutants resistant to clofazimine for WGS was isolated as described previously.[14] Genomic DNA from 10 clofazimine-resistant mutants, as well as the parent strain H37Rv, was sequenced using MiSeq (Illumina, Inc.) as described previously.[15] Single-nucleotide variants (SNVs) and insertions and deletions (InDels) ranging from 1 to 5 bp were sorted and called at a minimum coverage of 10 reads.

PCR and DNA sequencing

The clofazimine-resistant mutants were subjected to genomic DNA isolation as described previously.[15] The genomic DNA from clofazimine-resistant mutants isolated *in vitro* was then subjected to PCR amplification using *rv0678* primers Rv0678F (5′-TGCCTTCGGAACCAAAGAA-3′; 193 bp before start codon) and Rv0678R (5′-GACAACACGGTCACCTACAA-3′; 133 bp after stop codon) and the PCR products were sequenced as described previously.[15] The *rv1979* gene was PCR-amplified using primers 1979cF (5′-GCGGCGGAAATGAGTGT-3′) and 1979cR (5′-ATGCACGACGGCTTTATCA-3′). The *rv1979* PCR products were obtained using the same parameters as for PCR amplification of *rv0678* and sequenced as above to confirm the mutations in this gene in selected mutants (Table 1).

Table 1. Mutation analysis of 96 clofazimine-resistant mutants of *M. tuberculosis*

Gene	Nucleotide change	Amino acid change	Mutant count
rv0678	G193 deletion	65 codon shift	23
rv0678	G193 insertion[a]	65 codon shift	21
rv0678	C466T	R156[b]	11
rv0678	C364 insertion	122 codon shift	5
rv0678	A202G[a]	S68G	5
rv0678	T2C	start codon mutation	2
rv0678	T29 insertion	10 codon shift	1
rv0678	G58T	V20F	1
rv0678	C98A	T33N	1
rv0678	C107T	A36V	1
rv0678	G125A	W42[b]	1
rv0678	T128G	L43R	1
rv0678	G137A	C46Y	2
rv0678	A152G	Q51R	1
rv0678	C158T	S53L	1
rv0678	C176T	A59V	1
rv0678	G188A	S63N	1
rv0678	G194A	G65E	2
rv0678	G197T	G66V	1
rv0678	C226T	Q76[b]	1
rv0678	C251A	A84E	1
rv0678	G266T	R89L	1
rv0678	G269C	R90P	1
rv0678	A292 deletion	98 codon shift	1
rv0678	G304A	A102T	1
rv0678	C305T	A102V	2
rv0678	T341C	L114P	1
rv0678	T365C	L122P	1
rv0678	CGCTGGGC371–378 deletion	124 codon shift	1
rv0678	CG444–445 deletion	148 codon shift	1
rv1979c	T1052C	V351A	3[c]
rv2535c	G265T	E89[b]	1

[a]Mutation types that have been reported before.
[b]Indicates stop codon.
[c]Indicates one mutant had the *rv1979c* T1052C mutation only and two mutants had the *rv1979c* T1052C mutation, as well as *rv0678* G193ins.

Results

Isolation of mutants resistant to clofazimine

The cfu count showed that $\sim 2\times10^{7}$ *M. tuberculosis* bacteria were spread on plates containing different concentrations of clofazimine for mutant isolation. After 4 weeks of incubation, ~90 mutants were obtained on the plates containing 0.25 mg/L clofazimine, 6 mutants grew on the 0.5 mg/L plates and no mutants grew on plates containing 1 mg/L clofazimine. The mutation frequency of resistant mutants to 0.25 mg/L clofazimine was $\sim 5\times10^{-6}$. We then determined the MICs for the selected mutants and found that no mutants grew at clofazimine concentrations >1 mg/L.

WGS identified rv0678 as the most common mutated gene

We were able to isolate 96 *M. tuberculosis* H37Rv mutants resistant to clofazimine and chose 10 mutants for WGS to identify possible mechanisms of resistance. Genome sequence comparison of the resistant mutants and parent strain H37Rv found that *rv0678*, which encodes a transcriptional repressor, was mutated in all 10 of the resistant mutants. Six of the 10 mutants had a mutation at nucleotide position 193 of the *rv0678* gene, 4 had a G deletion and 2 had a G insertion, both causing a frameshift at this site.

Since the WGS analysis of the 10 mutants suggests that the *rv0678* mutation is the major mechanism of clofazimine resistance, to more effectively identify new mechanisms of clofazimine resistance we PCR-amplified and sequenced the *rv0678* gene in the remainder of the clofazimine-resistant strains. In total, we found that 93 of the 96 (~97%) clofazimine-resistant mutants had a mutation in *rv0678* (Table 1), demonstrating that *rv0678* mutation is the most prominent mechanism of resistance to

clofazimine. Consistent with its role as a repressor of efflux pump MmpL5, the mutations we identified in *rv0678* were primarily those [65/93 (70%) of all mutations] that inactivate the function of Rv0678 due to deletions, insertions causing frameshift mutations, or mutations that result in stop codons (Table 1). We found two mutation hot spots, at nucleotide positions 193 and 466. The nucleotide position 193 mutations, including both deletion and insertion at this position, accounted for 43.8% (42 out of 96 strains) of the mutations. The second hot spot occurred at nucleotide position 466, where 11.5% (11 out of 96 strains) had the C466T mutation. Insertion of C at nucleotide position 364 occurred in 5 of 96 mutants, whereas the A202G mutation, causing the amino acid change S68G, which was reported previously in a clofazimine-resistant mutant, occurred in 5 of 96 mutants (Table 1). The remaining 34 mutations were scattered along the entire *rv0678* gene with no apparent clustering. However, two clofazimine-resistant mutants, CT1-5 and CT3-5, did not have any mutations in the *rv0678* gene, indicating they harbour possible new mechanisms of resistance.

Identification of new genes associated with clofazimine resistance

To identify new mechanisms of clofazimine resistance in the two clofazimine-resistant mutants (CT1-5 and CT3-5) that did not harbour any *rv0678* mutations, we subjected the mutants to WGS. Comparative genome sequence analysis revealed that the CT1-5 mutant had a G265T mutation, leading to a stop codon at E89 in the *rv2535c* gene, which encodes a putative peptidase PepQ (Table 1). The other mutant, CT3-5, had a T1052C mutation, causing the amino acid change V351A in the gene *rv1979c*, which encodes a possible permease that might be involved in amino acid transport (Table 1). We also found two mutants with mutations in *rv0678*, one having a nucleotide G insertion at position 193 and one having a nucleotide G deletion at position 193 (Table 1) besides having the same *rv1979c* T1052C (V351A) mutation. Thus, we had three mutants with identical *rv1979c* T1052C (V351A) mutations; one mutant had only the *rv1979c* T1052C (V351A) mutation, but the two other mutants each had a G insertion and deletion at position 193 of the *rv0678* gene. It is of interest to note that the additional T1052C (V351A) mutation in *rv1979c* may confer a higher level of clofazimine resistance, as the two mutants with both *rv0678* mutations and the *rv1979c* mutation had a higher clofazimine MIC (1 mg/L) compared with the mutant with only the *rv1979c* mutation (Table S1, available as Supplementary data at *JAC* Online).

Discussion

A previous study that analysed a single clofazimine-resistant mutant identified C189A, leading to S63R mutation in *rv0678*.[10] In this study, to shed light on the relative importance of the *rv0678* mutation in clofazimine resistance and to identify new mechanisms of resistance, we analysed 96 clofazimine-resistant mutants and found 93 of them had *rv0678* mutations. The findings indicated that mutations in *rv0678* are the major mechanism of clofazimine resistance. Our study confirms and expands our understanding of mechanisms of resistance to clofazimine by identifying a spectrum of mutations in *rv0678* that cause clofazimine resistance. In addition, this study provides new insight into the mechanisms of resistance to clofazimine by identifying two new genes (*rv1979c* and *rv2535c*), which were previously not known to be associated with clofazimine resistance.

It is worth noting that the identical 193 G insertion mutation, which was the second most-common mutation (21/96) in our clofazimine-resistant mutants (Table 1), is also found in bedaquiline-resistant mutants isolated during MDR-TB treatment.[11] This finding, which is quite worrying, provides further evidence that resistance to clofazimine in *M. tuberculosis* will be cross-resistant to bedaquiline. Except for two mutations [193 G insertion mutation and A202G (S68G)] that were previously reported,[10,11] we identified 28 new mutations scattered along the entire *rv0678* gene (Table 1). The high diversity of scattered mutations is reminiscent of the diverse *pncA* mutations that cause pyrazinamide resistance.[16,17] InDels that cause frameshift and nonsense mutations or point mutations that result in stop codons were responsible for a large proportion (70%) of all mutations in *rv0678* (Table 1). This makes sense because all the mutations that abolish the repressor activity of Rv0678 will cause the overexpression of efflux pump MmpL5, which in turn causes clofazimine resistance. The crystal structure of Rv0678 demonstrated that it forms a dimer and binds to a 2-stearoylglycerol ligand.[18] This finding suggests that Rv0678 may be subject to regulation by metabolic products of the bacteria, which may affect the efflux activity of MmpL5 and susceptibility to clofazimine and bedaquiline. The mutation information identified in this study will be important for designing new molecular tests for rapid detection of clofazimine resistance and its cross-resistance to bedaquiline in future, as it is being more commonly used for treating TB and MDR-TB.

It is of interest to note that only low-level resistant *M. tuberculosis* mutants (<1 mg/L) could be isolated *in vitro* on 7H11 plates. More mutants were isolated on 0.25 mg/L clofazimine than at higher drug concentrations, and only five mutants were isolated on 0.5 mg/L clofazimine and no mutants could be isolated at 1 mg/L (C_{max}=0.47−0.7 mg/L) or higher clofazimine concentrations. This could be a desirable feature of this interesting drug, but it remains to be seen whether mutants resistant to >1 mg/L clofazimine could be isolated from patients as clofazimine is increasingly used to treat MDR-TB. Although several clinical isolates resistant to bedaquiline have been identified to have *rv0678* mutations with cross-resistance to clofazimine,[11,12] clinical isolates of *M. tuberculosis* resistant to clofazimine remain to be isolated and characterized.

We found that one mutant without the common *rv0678* mutation had a T1052C mutation in a new gene, *rv1979c*, causing the amino acid change V351A (Table 1). Rv1979c is a putative permease that might be involved in amino acid transport. It is possible that Rv1979c is involved in clofazimine transport or uptake, either directly or indirectly, to alter the physiology of the bacteria so as to be less susceptible to the effect of clofazimine. Future studies are needed to address these possibilities. In addition, it is of interest to note that Rv1979c is localized in the RD2 region and its deletion has been shown to be involved in virulence attenuation in BCG.[19] It remains to be tested whether resistance to clofazimine alters the virulence of *M. tuberculosis*.

It is intriguing that we found a stop codon mutation at E89 in a second new gene, *rv2535c*, involved in clofazimine resistance, inactivating the function of this protein. The exact function of Rv2535c is unknown, but it belongs to the peptidase family

M24B. While the exact function of Rv2535c remains to be determined, it is possible that clofazimine is a prodrug that may require activation by Rv2535c. Future studies are needed to determine the role of Rv2535c in clofazimine resistance in *M. tuberculosis*.

In summary, this study characterized a large number of mutants resistant to clofazimine and found mutations in *rv0678* to be the most prominent mechanism of clofazimine resistance. Our findings of mutation features associated with clofazimine resistance provide valuable information for the design of new molecular tests for the rapid detection of clofazimine resistance in the clinical setting. In addition, we discovered two new genes (*rv1979c* and *rv2535c*) associated with clofazimine resistance. Further studies are needed to address the role of *rv1979c* and *rv2535c* in clofazimine resistance and to understand the mechanisms of action of clofazimine.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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