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Unexpected high prevalence of resistance-associated *Rv0678* variants in MDR-TB patients without documented prior use of clofazimine or bedaquiline

Cristina Villellas[1], Nele Coeck[2], Conor J. Meehan[2], Nacer Lounis[1], Bouke de Jong[2], Leen Rigouts[2] and Koen Andries[1]*

[1]*Janssen Research and Development, Beerse, Belgium;* [2]*Institute of Tropical Medicine, Antwerp, Belgium*

*Corresponding author. Tel: +32-14602033; E-mail: kandries@its.jnj.com

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Objectives: Resistance-associated variants (RAVs) in *Rv0678*, a regulator of the MmpS5-MmpL5 efflux pump, have been shown to lead to increased MICs of bedaquiline (2- to 8- fold) and clofazimine (2- to 4-fold). The prevalence of these *Rv0678* RAVs in clinical isolates and their impact on treatment outcomes are important factors to take into account in bedaquiline treatment guidelines.

Methods: Baseline isolates from two bedaquiline MDR-TB clinical trials were sequenced for *Rv0678* RAVs and corresponding bedaquiline MICs were determined on 7H11 agar. *Rv0678* RAVs were also investigated in non-MDR-TB sequences of a population-based cohort.

Results: *Rv0678* RAVs were identified in 23/347 (6.3%) of MDR-TB baseline isolates. Surprisingly, bedaquiline MICs for these isolates were high (> 0.24 mg/L, $n = 8$), normal (0.03–0.24 mg/L, $n = 11$) or low (< 0.03 mg/L, $n = 4$). A variant at position −11 in the intergenic region *mmpS5–Rv0678* was identified in 39 isolates (11.3%) and appeared to increase the susceptibility to bedaquiline. In non-MDR-TB isolates, the frequency of *Rv0678* RAVs was lower (6/852 or 0.7%). Competition experiments suggested that rifampicin was not the drug selecting for *Rv0678* RAVs.

Conclusions: RAVs in *Rv0678* occur more frequently in MDR-TB patients than previously anticipated, are not associated with prior use of bedaquiline or clofazimine, and in the majority of cases do not lead to bedaquiline MICs above the provisional breakpoint (0.24 mg/L). Their origin remains unknown. Given the variety of RAVs in *Rv0678* and their variable effects on the MIC, only phenotypic drug-susceptibility methods can currently be used to assess bedaquiline susceptibility.

Introduction

Resistance-associated variants (RAVs) that lead to increased MICs of bedaquiline have been described in three genes of *Mycobacterium tuberculosis*: *atpE*, *Rv0678* and *pepQ*.[1–6] RAVs in *atpE*, a gene coding for a transmembrane protein of the ATP synthase, target of bedaquiline, lead to 8- to 133- fold increases in bedaquiline MIC and have been isolated *in vitro* upon exposure to bedaquiline, but they have so far not been observed in clinical isolates from patients treated with bedaquiline.[7] RAVs in *pepQ*, leading to 4-fold increases in bedaquiline and clofazimine MICs, have been isolated from mice treated with bedaquiline. RAVs in *Rv0678*, a gene regulating the expression of the MmpS5-MmpL5 efflux pump, lead to 2- to 8- fold increases in bedaquiline MIC and 2- to 4-fold increases in clofazimine MIC.[1] They have been isolated *in vitro* upon exposure to clofazimine[3] or bedaquiline,[1] and have also been observed in some post-baseline isolates of patients treated with bedaquiline.[7] Although RAVs in *Rv0678* lead to increased MICs of clofazimine, it is not clear whether the use of clofazimine in TB patients can select for these mutants. The prevalence of these *Rv0678* RAVs in clinical isolates and their impact on bedaquiline MICs and treatment outcomes are important factors to take into account in bedaquiline treatment guidelines. Upon the surprising discovery of an *Rv0678* RAV in a baseline isolate from a patient without documented prior use of clofazimine or bedaquiline, we studied the prevalence of these RAVs in clinical isolates of MDR-TB and non-MDR-TB patients.

Materials and methods

MDR clinical isolates

Baseline (day 1) *M. tuberculosis* isolates from participants in Phase 2b studies C208[8] and C209[7] were included. On day 1 there were 374 isolates available, of which 363 had bedaquiline MICs available, and, out of these, DNA

was available for 359 isolates (96%), which had their *Rv0678* gene sequenced. Only seven of these patients had documented prior use of bedaquiline of clofazimine in their medical history.

Susceptibility testing

Bedaquiline MICs for C208 and C209 MDR-TB isolates were determined on Middlebrook 7H11 agar at 0.004, 0.008, 0.015, 0.03, 0.06, 0.12, 0.24, 0.48, 1.0, 2.0, 4.0 and 8.0 mg/L as described previously.[9] Each new batch of 7H11 medium had the H37Rv reference strain (BCCM/ITM 083715) tested as quality control, with an acceptable MIC range of 0.03–0.12 mg/L. The MIC was determined as the lowest concentration of the antibiotic that resulted in 99% growth inhibition.

Susceptibility of BCA8, an H37Rv-derived *Rv0678* mutant,[1] to TB drugs other than pyrazinamide was determined in 7H9 broth by the resazurin microtitre assay (REMA) as previously described.[10] The percentage of growth in each well was calculated using the fluorescence of the growth control minus the fluorescence of the non-growth control as 100% growth. The MIC_{50} was defined as the lowest concentration of antibiotic that resulted in 50% inhibition of growth. The average of the MIC_{50}s was taken after log transforming all individual MIC_{50}s, including the censored ones. The censor added to the average was based on the relative occurrence of the censor in the individual data. When the censor occurred in less than one-third of the data, the censor was omitted. When the censor occurred in more than two-thirds of the data, the censored was added to the average. When the censor occurred between one-third and two-thirds of the data the censored was used but an equals sign was added. To calculate the significance of the fold change, a one-sided unpaired *t*-test was performed with unequal variance. Pyrazinamide resistance testing was done using the MGIT-PZA kit (Becton Dickinson) as per the manufacturer's instructions.

Rv0678, atpE and pepQ sequencing

A DNA fragment containing *Rv0678* and part of the intergenic region between *mmpS5* and *Rv0678* of C208 and C209 MDR-TB isolates was amplified by PCR using primers CV010 and CV017 or 916R20 and 30F22 (Table S1, available as Supplementary data at *JAC* Online). The *atpE* and *pepQ* genes were amplified using primers atpEforward with atpEreverse, and pepQforward with pepQreverse, respectively, (Table S1) in the eight baseline isolates with MICs above the breakpoint (> 0.24 mg/L).[11] The PCR products sequenced with the same primers used for amplification. For analysis of the sequences, *Rv0678* sequence from *M. tuberculosis* H37Rv was taken as a reference[12] (http://tuberculist.epfl.ch).

Data mining of non-MDR clinical isolates

A total of 941 isolates from the Hamburg non-MDR-TB cohort[13] were mined to look for mutations in the same region. These samples were isolated between 2004 and 2012 and sequenced using the Illumina platforms with read lengths of 100 bp. SNP calling was undertaken using the Snippy v2.9[14] pipeline, which employs BWA (read-quality cleaning and mapping functions), SAMtools and FreeBayes. The genome of strain H37Rv (NC-000962.3) was used as a reference. Drug resistance and lineage assignment was determined from the genomes using the PhyResSE resistance list version 27.[15]

TB lineage analysis

TB lineage analysis for the clinical trial isolates was done by a real-time PCR assay targeting lineage-specific SNPs as described by Stucki *et al.*[16] with modified probes and primers for lineage 2 (Table S2). Isolates with an unclear SNP profile were tested by spoligotyping using the MIRU-VNTRplus online application (http://www.miru-vntrplus.org/MIRU/index.faces).

Competition experiments

Two *M. tuberculosis* H37Rv-derived strains were used: H37Rv-rpoB, carrying a mutation in the *rpoB* gene (H526Y); and CV37-rpoB, carrying mutations in *rpoB* (H526Y) and *Rv0678* (*IS*6110 nt 104). These strains were transformed with pND235,[17] a plasmid containing green fluorescent protein (GFP) (λem 475 nm, λex 525 nm), and pND239,[18] a plasmid containing DsRed2 (λem 575 nm, λex 632 nm), in order to generate fluorescence-marked strains (Table S1). To rule out any effect of the plasmids on the fitness of bacteria, competition experiments were done in two pairs: H37Rv-rpoB-pND235 (GFP marked) with CV37-rpoB-pN239 (DsRed2 marked); and H37Rv-rpoB-pND239 (DsRed2 marked) with CV37-rpoB-pN235 (GFP marked).

Competition experiments were adapted from Gullberg *et al.*[19] Cultures were grown in 7H9/10% OADC/0.05% Tween broth to the logarithmic phase and diluted to OD = 0.1. These were further diluted 1:200 to obtain a final bacterial inoculation density of $\sim 5 \times 10^4$ cfu/mL per strain. Two independent flasks were inoculated with: (i) H37Rv-rpoB-pND235 and CV37-rpoB-pND239; and (ii) H37Rv-rpoB-pND239 and CV37-rpoB-pND235. Aliquots (5 mL) from each culture were put in 10 flasks; rifampicin was added to 8 of them (concentrations: 200, 100, 50, 25, 12.5, 6.25, 0.625 and 0.0625 mg/L) and 2 remained antibiotic-free. After incubation for 1 week at 37 °C, a 500 μL aliquot from all cultures was washed with 200 μL of PBS, inactivated for 90 min at 4 °C in 400 μL of 4% paraformaldehyde[20] and finally resuspended in 200 μL of PBS. Serial decimal dilutions were analysed using a fluorescence-activated cell sorter in an FACS Fortessa. A minimum of 10^4 cells were counted per sample. The percentages of GFP-containing and DsRed2-containing bacilli were determined in each sample and the ratio of CV37-rpoB to H37Rv-rpoB was calculated for each condition. On the same day, every culture was passaged at a dilution of 1:1000 to a new flask containing the same concentration of rifampicin. These steps of sampling and passage were repeated every week for 3 weeks.

Results

MDR-TB clinical isolates at baseline contain RAVs in Rv0678, not always linked to high bedaquiline MICs

Interpretable *Rv0678* sequencing results and bedaquiline MICs were available for 347 out of 359 investigated baseline isolates from MDR-TB isolates (Table 1). Their MICs ranged from 0.004 to 1.0 mg/L, with a mean MIC of 0.06 mg/L. The majority (296/347, 85.3%) had normal MICs (0.03–0.24 mg/L) (typically seen for the H37Rv reference strain), a smaller proportion (43/347, 12.4%) had low MICs (≤ 0.015 mg/L) and 8/347 (2.3%) isolates had an MIC > 0.24 mg/L, the provisional breakpoint for bedaquiline on 7H11 agar.[11]

An overview of all the *Rv0678* RAVs is presented in Figure 1 and Table S3. The higher MICs could all be explained by RAVs in *Rv0678* – no RAVs were found in *atpE* or *pepQ* genes of these isolates. However, 15 additional baseline isolates (4.3%) had similar *Rv0678* RAVs that did not result in a high bedaquiline MIC. Taken together, 6.6% (95% CI 4.0%–9.2%) of MDR-TB baseline isolates had an RAV in the *Rv0678* coding sequence. *Rv0678* RAVs were not identified in any of the seven patients that had failed previous therapy with a regimen containing clofazimine prior to enrolment in the bedaquiline trial.

Intergenic mutations at positions −44 and −59, outside of the hypothetical binding site of *Rv0678* described by Milano *et al.*[21] were observed in < 1% of the isolates and were not associated with high bedaquiline MICs. Intergenic mutations at position −11 were observed in 39 out of the 347 isolates (11%); in 2 out of 39 this mutation appeared simultaneously with another non-silent RAV in *Rv0678*. The 37 isolates with only a mutation at position

Table 1. MICs for and mutations of MDR-TB baseline clinical isolates

	Number of isolates with bedaquiline MIC (mg/L)									
Rv0678 locus	1.000	0.480	0.240	0.120	0.060	0.030	0.015	0.007	0.004	Total
Non-silent mutation	1	5	2	3				1	1	13
WT + non-silent mutation	1			1	3	1				6
WT + non-silent mutation + mutation in position −11							2			2
WT + non-silent mutation + silent mutation					1					1
Non-silent mutation + mutation in position −13		1								1
Intergenic mutation in position −11 or intergenic mutation in position −11 + *Rv0678* silent mutation						7	28		2	37
Intergenic mutation, not −11			1		1					2
Silent mutations or mixture of WT + silent				1	2	1				4
WT			2	48	153	69	7		2	281
Total analysed	2	6	5	53	160	78	37	1	5	347

The numbers of isolates with high (> 0.24 mg/L), normal (0.03–0.24 mg/L) and low (< 0.03 mg/L) bedaquiline MICs are indicated for every type of mutation in the *Rv0678* gene and the intergenic region *mmpS5–Rv0678* .
Non-silent mutations refer to non-synonymous mutations and small insertions or deletions in the *Rv0678* coding region.

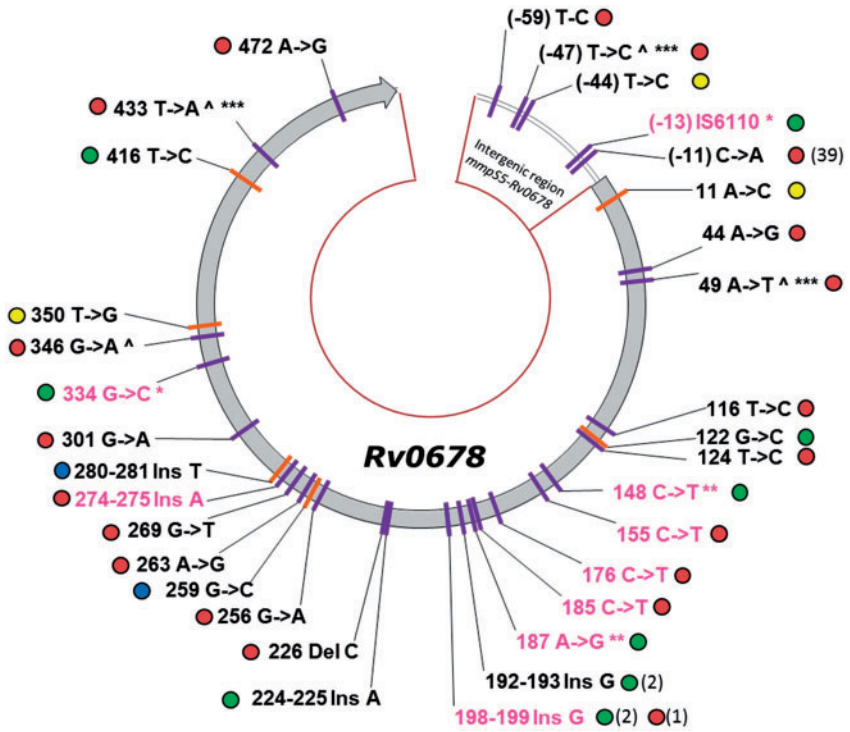

Figure 1. Mutations in *Rv0678* and the intergenic region *mmpS5–Rv0678* and lineage of their respective isolates. All the different DNA mutations found in C208 and C209 baseline isolates (MDR, purple markers) and in the Hamburg cohort (non-MDR, orange markers) are shown. Silent mutations are not shown. Mutations in isolates with high MICs are coloured in pink. The circle next to every mutation indicates the lineage of the isolate: blue, lineage 1; red, lineage 2; yellow, lineage 3; and green, lineage 4. When the mutation was found in more than one isolate, the number of isolates carrying that mutation is indicated in brackets. Some isolates carry more than one mutation, and the mutations found in the same isolate are indicated with the same symbol (*, ** or ***). The mutations co-existing with the mutation in position −11 are marked with the symbol^.

−11 were associated with low to moderate bedaquiline MICs (mean MIC 0.015 mg/L). An *IS*6110 insertion at position −13 in the intergenic region was present in combination with a missense mutation in *Rv0678* in one isolate with a high MIC. The heterogeneous nature of clinical isolates explains why in some cases mixtures of WT sequences and RAVs were observed (nine isolates, 2.6%). Silent mutations in *Rv0678* were observed in 1% of the isolates.

To investigate whether the presence of *Rv0678* RAVs at baseline was associated with a specific lineage of *M. tuberculosis*, the presence of lineage-specific SNPs was analysed.[16] The majority of the isolates belonged to lineage 2 (167/347, 48.1%) and lineage 4 (159/347, 45.8%), and only a few to lineage 1 (13/347, 3.7%) and lineage 3 (8/347, 2.3%). Baseline isolates carrying *Rv0678* RAVs, including the intergenic region, were more abundant in lineage 2 (54/167, 32.3%). Interestingly, all the isolates containing the same mutation at position −11 in the intergenic region *mmpS5–Rv0678* belonged to the Beijing family of lineage 2, which suggests that all these might be phylogenetically close. Out of the 54 isolates with *Rv0678* RAVs, 19 contained a missense mutation or an intergenic mutation different from −11 C→A in *Rv0678*, either alone or in a mixture. *Rv0678* RAVs were less frequent in lineage 3 (1/8, 12.5%) and lineage 4 (7/159, 4.4%). None of the 13 isolates belonging to lineage 1 had *Rv0678* RAVs.

Out of the eight isolates with high baseline MICs (> 0.24 mg/L), five belonged to lineage 2 and three belonged to lineage 4. Low bedaquiline MICs were more frequent in lineage 2 (34/43) and normally associated with the *Rv0678* RAV in position −11.

Non-MDR-TB isolates

Sequencing data were available for 941 isolates from non-MDR-TB patients of the Hamburg cohort. Read depth coverage resulting from mapping to the reference H37Rv genome averaged at 154 × (range 34 × to 740 ×) across isolates. Based on genomic SNP data, 852 isolates (90.5%) were pan-susceptible to all drugs included in the PhyResSE resistance list (fluoroquinolones, ethionamide, rifampicin, streptomycin, linezolid, amikacin, kanamycin, ciprofloxacin, isoniazid, pyrazinamide, *p*-aminosalicylic acid and ethambutol). Isolates with predicted drug resistance were usually resistant to isoniazid (66 isolates). Six isolates had RAVs in *Rv0678* (0.7% prevalence, 95% CI 0.1%–1.2%), although no other drug resistance was detected in these isolates. These isolates were not clustered in a transmission chain (i.e. > 12 SNPs from each other[22]) and were from lineages 1 (2/54 isolates), 3 (2/65 isolates) and 4 (2/704 isolates) (Figure 1).

Cross-resistance of Rv0678 mutants with other TB drugs and potential selection pressures

The high prevalence of *Rv0678* RAVs in baseline samples from patients without prior treatment with bedaquiline or clofazimine suggests that there is selection pressure favouring *Rv0678* RAVs. The fact that many *Rv0678* RAVs are present in a mixture with WT *Rv0678* suggests that this selection pressure is mild, which is in accordance with a moderate fold increase in the MIC of the drug. We determined the susceptibility of BCA8, an *Rv0678* mutant strain derived from H37Rv,[1] to a panel of first- and second-line TB drugs to identify additional cross-resistance with bedaquiline via the same mechanism (Table 2). Bedaquiline and clofazimine had increased MICs for the *Rv0678* mutant strain. Telithromycin and rifampicin also displayed slightly increased MICs for the *Rv0678* mutant (5- and 3-fold, respectively), although the results were not statistically significant. We hypothesized that *Rv0678* RAVs, leading to overexpression of the MmpL5 efflux pump, could benefit the survival of MDR strains in the presence of rifampicin (see the Discussion section). To test this hypothesis, we looked at the competitive advantage of the double mutant *Rv0678-rpoB* over the single *rpoB* mutant in the presence of different concentrations of rifampicin (ranging from 0.06 to 200 mg/L) through several generations. If the double mutant had a competitive advantage in the presence of rifampicin, then this population would be enriched in every generation. As can be observed in Figure S1, we found the opposite effect: the population of the double mutant *Rv0678-rpoB* decreased in every passage. Rifampicin is apparently not able to select for *Rv0678* RAVs in an *rpoB* mutant background, and is therefore unlikely to be responsible for the higher prevalence of *Rv0678* RAVs in MDR baseline isolates.

Discussion

Of the MDR-TB isolates, 2.3% (8/347) had bedaquiline MICs > 0.24 mg/L. All high baseline MICs could be explained by RAVs in *Rv0678*, and none of these isolates had an RAV in *atpE* or *pepQ*. RAVs in *Rv0678* were not only identified in isolates with high baseline MICs: 4.3% of the isolates had similar RAVs (some with insertions or deletions), not resulting in bedaquiline MICs exceeding the bedaquiline breakpoint, suggesting a role of additional genes in determining the bedaquiline MIC. In total, RAVs in *Rv0678* were observed in 6.6% of the MDR-TB isolates and they were associated with bedaquiline MICs exceeding the breakpoint in only 1 out of 3 cases (8 out of 23).

Intergenic mutations were observed in < 1% of the isolates and generally were not associated with high bedaquiline MICs. RAVs in the intergenic region between *Rv0678* and *mmpS5* at positions −9 and −10 in the hypothetical binding site of Rv0678 were previously shown to increase the expression of the MmpL5 efflux pump.[21] Only one mutation was found at this binding site, and it was combined with another mutation in the gene itself, both probably contributing to the high bedaquiline MIC for the isolate. RAVs at −44 and −59, outside of the *Rv0678* binding site, were identified in < 1.0% of the isolates, but did not result in high bedaquiline MICs (0.06–0.24 mg/L). RAVs at intergenic position −11 have not been described before and were observed in 10.7% of the 347 MDR-TB isolates in our sample. Interestingly, RAVs at this position were only observed in isolates from (several locations in) South Africa and they all belong to the W-Beijing lineage. They were associated with low to moderate bedaquiline MICs (0.004–0.03 mg/L, mean MIC 0.015 mg/L).

In 2.6% of the isolates we found a mixture of WT and mutant *Rv0678*. Mixtures could be the result of either spontaneous mutations that were further selected, or a mixed infection with two strains. Since the Sanger sequencing technique does not provide this information, we performed MIRU-VNTR typing for the mixtures and found no evidence of mixed infection, so the *Rv0678* mutants were a subpopulation that was selected within the patient.

The prevalence of *Rv0678* RAVs in pan-susceptible TB isolates from the population-based Hamburg cohort was 9-fold lower compared with the MDR-TB cohort, with only 6 sequences out of the 852 (0.7%) having a polymorphism in *Rv0678*. Bedaquiline MICs were not available for this data set.

The high prevalence of *Rv0678* RAVs in baseline isolates of MDR-TB patients is surprising, given that none of these patients had been exposed to bedaquiline; nor was there any evidence for prior use of clofazimine in these patient's medical histories (except for seven subjects). Although one cannot exclude that some of these

Table 2. MIC_{50}s of a panel of first- and second-line TB drugs for H37Rv and BCA8

Compound	MIC_{50} (mg/L) (*n*)		Fold change in MIC_{50} BCA8/H37Rv	*P*
	H37Rv	BCA8		
Bedaquiline base	0.04 (11)	0.31 (10)	7.77*	<0.0004
Bedaquiline fumarate	0.04 (6)	0.28 (5)	6.74*	<0.0004
Clofazimine	0.11 (7)	0.38 (5)	3.52*	<0.0008
Thioridazine	6.44 (6)	10.09 (2)	1.57*	0.04084
Telithromycin	2.28 (7)	11.26 (5)	4.94	>0.05
Rifampicin	0.01 (13)	0.02 (10)	2.86	>0.05
Amoxicillin/clavulanic acid 2/1	5.73 (6)	9.61 (4)	1.68	>0.05
Rifapentin	≤ 0.005 (7)	0.01 (5)	≥ 1.57	>0.05
Ethambutol	1.81 (7)	2.38 (5)	1.31	>0.05
Moxifloxacin	0.07 (7)	0.08 (5)	1.24	>0.05
Erythromycin	5.26 (7)	≥ 6.36 (5)	≥ 1.21	>0.05
Clarithromycin	0.6 (12)	0.66 (10)	1.09	>0.05
Rifabutin	≤ 0.003 (6)	0.003 (2)	≥ 1.01	>0.05
Amikacin	0.39 (6)	0.36 (5)	0.93	>0.05
Ofloxacin	0.32 (7)	0.29 (5)	0.92	>0.05
Terizidone	4.32 (6)	3.76 (2)	0.87	>0.05
Levofloxacin	0.22 (13)	0.18 (10)	0.79	>0.05
Linezolid (batch 2)	0.37 (7)	0.29 (5)	0.78	>0.05
Capreomycin	0.61 (7)	0.45 (5)	0.74	>0.05
Linezolid (batch 1)	0.47 (6)	0.30 (5)	0.62	>0.05
p-Aminosalicylic acid	0.02 (7)	0.01 (5)	0.5	>0.05
Streptomycin	0.33 (6)	0.16 (2)	0.48	>0.05
Isoniazid	0.12 (7)	0.05 (4)	0.46	>0.05
Protionamide	2.11 (5)	0.82 (1)	0.39	>0.05

The MIC_{50}s for the WT (H37Rv) and an *Rv0678* mutant (BCA8) are shown.
The number of times that the compound was tested is indicated in parentheses.
The MIC_{50} fold changes are calculated based on unrounded values.
Fold changes based on censored values become themselves censored.
The MIC_{50} fold changes that are statistically significant ($P < 0.05$) are marked with an asterisk.
Pyrazinamide was tested using the MGIT-PZA kit; both BCA8 and H37Rv were susceptible to this drug.

patients were originally infected with strains already resistant to clofazimine, several factors argue against this possibility. Firstly, *Rv0678* RAVs were not identified in any of the seven patients that had failed previous therapy with a regimen containing clofazimine prior to enrolment in the bedaquiline study. Secondly, the prevalence of *Rv0678* RAVs was not higher in treatment-experienced MDR-TB patients or in pre-XDR- and XDR-TB patients.

Overall, *Rv0678* RAVs lead to low-level resistance, and the effect on treatment outcome is not clear. Several letters and papers reported on a single patient developing an *Rv0678* RAV while failing bedaquiline therapy and suggested a causal link between the RAV and the treatment failure,[23–25] but in a clinical trial 12 patients whose isolates had acquired an *Rv0678* RAV at the interim analysis were as likely to have converted at endpoint as those that had not developed an *Rv0678* RAV.[7] Although these data are limited, they do not suggest that increases in bedaquiline MICs on treatment and due to *Rv0678* RAVs have a negative effect on treatment response. There was also no clear relationship between bedaquiline baseline MIC and culture conversion at the endpoint, although more efficacy data are needed for patients with baseline MICs above the provisional breakpoint. Efficacy studies in a mouse model of TB infection indicate that bedaquiline treatment is still bactericidal for such isolates, although to a lesser extent compared with the effect seen when treating an isolate without an *Rv0678* RAV.[1]

The much lower frequency of *Rv0678* RAVs in sequences from non-MDR-TB patients suggests a role of prior exposure to TB drugs. As all RAVs described in this article were isolated from patients prior to the introduction of bedaquiline, prior exposure to clofazimine was the most obvious hypothesis to explain their origin. However, none of the seven patients *with* prior documented use of clofazimine in this study had developed RAVs in *Rv0678*, and use of clofazimine in patients has not yet been reported to select for *Rv0678* RAVs by other investigators. In view of these findings, prior exposure to clofazimine should not automatically lead to exclusion of bedaquiline treatment. *Rv0678* RAVs did not occur more frequently in treatment-experienced MDR-TB isolates or in isolates from pre-XDR- or XDR-TB patients, providing further evidence against a role of clofazimine.

To rule out that other TB drugs than clofazimine were responsible for the selection of *Rv0678* RAVs, an H37Rv isolate susceptible to first- and second-line anti-TB drugs, yet overexpressing the

MmpL5 efflux pump, was used to assess any cross-resistance with a large panel of TB drugs. The only MICs that were significantly increased (> 3-fold, $P < 0.05$) in this strain were those of bedaquiline and clofazimine. Rifampicin turned out to be the most suspicious candidate of the list, being a first-line drug with a slightly increased MIC (2.9-fold) in the mutant overexpressing the efflux pump. In addition, increased expression of this efflux pump was described in a rifampicin-resistant (*rpoB* mutant) strain upon exposure to subinhibitory levels of rifampicin.[26] We therefore investigated whether rifampicin could select for *Rv0678* mutations in *rpoB* mutants by assessing the competitive advantage of the double mutant *Rv0678-rpoB* over the single *rpoB* mutant, in the presence of different concentrations of rifampicin. Since the double mutant did not grow better than the single mutant in the presence of the drug, rifampicin is unlikely to be responsible for the high prevalence of *Rv0678* RAVs in MDR-TB baseline isolates.

The natural function of MmpS5/MmpL5 efflux pumps is to export siderophores.[27] Expression of the efflux pump is down-regulated in high iron conditions,[28] and one can expect up-regulation in low iron conditions. We checked whether the prevalence of *Rv0678* RAVs was higher in isolates from female patients, but this did not appear to be the case. Iron-limiting conditions are also thought to exist in granulomatous lesions of TB. The origin of these 'naive' *Rv0678* RAVs is therefore elusive at this point in time.

There was no evidence for *Rv0678* RAVs being linked to specific TB lineages, as they were observed in lineages 2, 3 and 4. In contrast, RAVs in the intergenic region at position −11 were exclusively observed in W-Beijing isolates.

Our study has several implications for bedaquiline DST development. The absence of an algorithm to predict bedaquiline MICs based on specific *Rv0678* RAVs limits the value of sequencing this specific gene—RAVs in *Rv0678* were associated with bedaquiline MICs exceeding the breakpoint in only 8 out of 23 cases. Sequencing *Rv0678* could be useful to rule out resistance to bedaquiline, but not to rule in resistance. Phenotypic DST tests remain the method of choice to monitor bedaquiline susceptibility

The prevalence of high baseline MICs of bedaquiline in 2.3% of patients without prior exposure to bedaquiline or clofazimine is worrisome and further surveillance studies are required to explain their origin, and to generate more data on their impact on treatment outcomes. The frequency of target-based (*atpE*) and non-target-based (*Rv0678*) RAVs for bedaquiline was similar in a set of *in vitro* selected isolates,[4] but the latter are more likely to emerge first, as efflux-based mutations generally lead to lower levels of resistance (2- to 16-fold higher MICs) than target-based mutations (16- to 1000-fold higher MICs). This increases the likelihood that non-target-based resistance will be used as a stepping stone eventually leading to higher-level, target-based resistance in patients on a failing treatment regimen.

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Transparency declarations

C. V., N. L. and K. A. are employees of Janssen Pharmaceutica. N. L. and K. A. hold shares of Johnson and Johnson. All other authors: none to declare.

Supplementary data

Tables S1 to S3 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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