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Cross-Resistance between Clofazimine and Bedaquiline through Upregulation of MmpL5 in *Mycobacterium tuberculosis*

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The antileprosy drug clofazimine is also of interest for the treatment of multidrug-resistant tuberculosis. To understand possible resistance mechanisms, clofazimine-resistant *Mycobacterium tuberculosis* mutants were isolated *in vitro*, and, unexpectedly, found to be cross-resistant to bedaquiline. Mutations in the transcriptional regulator Rv0678, with concomitant upregulation of the multisubstrate efflux pump, MmpL5, accounted for this cross-resistance. Mutation in Rv0678 should therefore be considered a confounding factor for the treatment of tuberculosis with clofazimine or bedaquiline.

Clofazimine, a riminophenazine, is a standard component of the multidrug therapy used for the treatment of leprosy and has contributed to the cure of 16 million patients. In recent years, due to the spread of multidrug-resistant *Mycobacterium tuberculosis* strains there has been renewed interest in the use of clofazimine for treatment of multidrug-resistant tuberculosis (1). Clofazimine has been elegantly demonstrated in *M. tuberculosis* to be a prodrug, which is reduced by NADH dehydrogenase (Ndh2), and then upon spontaneous reoxidation by O_2, to release reactive oxygen species (ROS) (2). As there is no specific target for ROS, resistance to clofazimine is rare and to our knowledge has never been characterized.

With the aim of further understanding the mechanism of action of resistance of clofazimine, we sought to determine the means by which *M. tuberculosis* strain H37Rv can develop resistance to the compound. Resistant mutants were selected by plating H37Rv (100 μl at an optical density at 600 nm [OD_{600}] of 1) on Middlebrook 7H11 medium containing clofazimine at concentrations only just greater (4×) than its MIC (solid clofazimine MIC, 0.25 μg/ml; isolation at 1 μg/ml). Following the passage of an isolated colony ($H37Rv_{CFZ\text{-}R1}$) in the absence of antibiotic pressure, resistance to clofazimine was maintained (4- to 8-fold), as determined using the resazurin reduction microplate assay (REMA) (3) (Fig. 1), suggesting the involvement of a genetic resistance mechanism. To evaluate the specificity of the mechanism of resistance, the susceptibility of $H37Rv_{CFZ\text{-}R1}$ was determined for a panel of antituberculosis drugs. While the MIC of $H37Rv_{CFZ\text{-}R1}$ for rifampin, PA-824, isoniazid, and moxifloxacin remained unchanged, marked cross-resistance was observed with bedaquiline (4- to 8-fold) and, to a much lesser extent, to the azole drug econazole (Fig. 1 and Table 1).

To identify the genetic mutation causing the clofazimine-bedaquiline cross-resistance, the genomes of H37Rv and $H37Rv_{CFZ\text{-}R1}$ were subjected to whole-genome sequencing using Illumina technology. Bioinformatics analysis (as described in reference 4) identified a nonsynonymous single nucleotide polymorphism in gene *rv0678* (c189a leading to S63R) in $H37Rv_{CFZ\text{-}R1}$, while *atpE* contained no mutations. The presence of this mutation was confirmed independently by Sanger sequencing. A second *de novo* round of mutant isolation yielded five further clofazimine-resistant mutants. Sanger sequencing of *rv0678* from these five isolates showed that four of them carried the same c189a mutation identified in $H37Rv_{CFZ\text{-}R1}$, and one ($H37Rv_{CFZ\text{-}R2}$) harbored a novel c400t mutation in *rv0678* that results in a premature stop codon (R134stop).

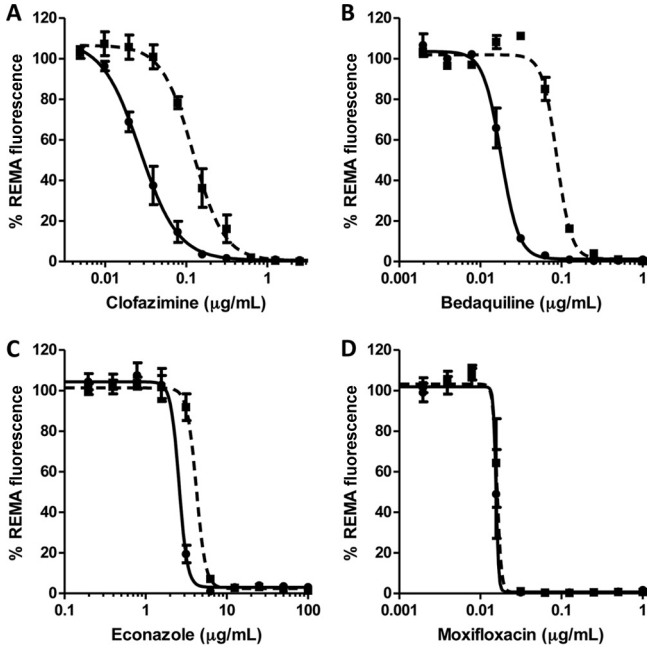

FIG 1 Drug susceptibility of MTB H37Rv (solid circles with solid lines) and $H37Rv_{CFZR1}$ (solid squares with dashed lines) to clofazimine (A), bedaquiline (B), econazole (C), and moxifloxicin (D) as measured by the resazurin reduction assay.

Rv0678 is a transcriptional repressor with a winged helix DNA binding domain that has been shown to bind to a palindromic

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TABLE 1 Drug susceptibility profiles of clofazimine-resistant and wild type *M. tuberculosis*

Strain	*rv0678* sequence characteristic(s)	MIC (μg/ml) of:			
		Clofazimine	Bedaquiline	Econazole	Moxifloxacin
H37Rv	WT[a]	0.312	0.125	5	0.031
$H37Rv_{CFZ-R1}$	c189a (S63R)	1.25	0.5	10	0.031
$H37Rv_{CFZ-R2}$	c400t (R134stop)	1.25	0.5	10	0.031
H37Rv::pMV261	WT	0.312	0.125	5	0.031
H37Rv::*rv0678*	WT	0.312	0.125	5	0.031
H37Rv::$rv0678_{(S63R)}$	WT and S63R	0.625	0.25	5	0.031

[a] WT, wild type.

sequence located in the intergenic region between *rv0678* and the neighboring genes, *mmpS5* and *mmpL5* (5). Numerous mutations in Rv0678 were found to lead to derepression of this locus with subsequent transcriptional upregulation of all three genes (*rv0678*, *mmpS5*, and *mmpL5*) (5). Here, using quantitative PCR (qPCR), we confirmed that $H37Rv_{CFZ-R1}$ carrying $Rv0678_{(S63R)}$ displays 11.6- and 11.2-fold-increased expression of *mmpL5* and *rv0678*, respectively, relative to wild-type H37Rv.

To confirm genetically the role of $Rv0678_{(S63R)}$ in resistance to clofazimine and bedaquiline, $rv0678_{(S63R)}$ was expressed from the *hsp60* promoter in the integrative vector pMV261 in *M. tuberculosis* strain H37Rv. Drug susceptibility evaluation by REMA clearly showed that expression of $Rv0678_{(S63R)}$ in H37Rv leads to partial resistance to both clofazimine and bedaquiline (Table 1). Partial, and not complete, resistance is likely because the chromosomal wild-type *rv0678* gene is still expressed. Ectopic overexpression of $Rv0678_{(S63R)}$ in $H37Rv_{CFZ-R1}$ had no further impact on drug susceptibility.

MmpL proteins (6) are multisubstrate efflux pumps that are part of the family of "resistance, nodulation, and cell division" (RND) proteins (7). MmpL5 is one of 13 such MmpL proteins found in *M. tuberculosis*, and together with MmpL4 has been shown to be involved in the export of siderophores (mycobactins and carboxymycobactins) for the bacterial acquisition of iron (8, 9). MmpL5 by itself has been found to be dispensable in *M. tuberculosis* (7), although removal of both MmpL5 and MmpL4 has a detrimental impact on both bacterial virulence and viability *in vivo* (8). Mutations in *rv0678*, with subsequent upregulation of *mmpS5* and *mmpL5*, have been shown to lead to resistance to azoles (5), as is also seen in the present study with resistance to econazole (Fig. 1). Resistance to azoles was demonstrated to be through the active efflux of these compounds by MmpL5, suggesting that clofazimine and bedaquiline may also be substrates for this multisubstrate efflux pump. This hypothesis is also supported by data demonstrating that the general inhibitor of efflux, verapamil, potentiates bedaquiline activity in *M. tuberculosis* (10). Nevertheless, it is intriguing that MmpL5 seems to affect in particular compounds acting on or affected by the electron transport chain (azoles, clofazimine, and bedaquiline). In addition, bedaquiline has been shown to act synergistically with both pyrazinamide *in vivo* (11) and benzothiazinones *in vitro* (12, 13), and a possible source of this synergy may be through modulation of efflux pumps, such as MmpL5.

Bedaquiline is currently one of the most promising novel antituberculosis drug candidates (14). In previous work with bedaquiline-resistant H37Rv mutants isolated *in vitro*, it was found that 15 out of 53 resistant mutants carried mutations in *atpE*, the gene that encodes the target subunit of bedaquiline in ATP synthase (15, 16). Nonetheless, the remaining 38 resistant mutants did not harbor mutations in *atpE*, and their resistance remains unexplained. These "off-target" genetic mutants typically showed a 2- to 16-fold level of increased resistance, a similar magnitude to that seen here in strains with mutations in *rv0678*. In the absence of data explaining non-*atpE*-mediated bedaquiline resistance, it remains to be determined whether mutations in *rv0678* are involved and whether these mutants display cross-resistance to clofazimine. The Global Alliance for TB Drug Development is currently conducting clinical trials to evaluate the activity of both clofazimine and bedaquiline in combination regiments for the treatment of tuberculosis. To date, very little is known about the potential sources of drug resistance in such regimens; however, mutations in *rv0678* should be taken into consideration as a possible confounding factor. An additional intriguing point is the absence of the *rv0678-mmpS5 mmpL5* locus in *Mycobacterium leprae*, and this may explain why leprosy is so effectively treated with clofazimine and resistance has never been encountered. In light of these findings, Rv0678 will be given the name MmpR5 (Mycobacterial membrane protein repressor).

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REFERENCES

1. **Van Deun A, Maug AK, Salim MA, Das PK, Sarker MR, Daru P, Rieder HL.** 2010. Short, highly effective, and inexpensive standardized treatment of multidrug-resistant tuberculosis. Am. J. Respir. Crit. Care Med. **182:** 684–692. http://dx.doi.org/10.1164/rccm.201001-0077OC.
2. **Yano T, Kassovska-Bratinova S, Teh JS, Winkler J, Sullivan K, Isaacs A, Schechter NM, Rubin H.** 2011. Reduction of clofazimine by mycobacterial type 2 NADH:quinone oxidoreductase: a pathway for the generation of bactericidal levels of reactive oxygen species. J. Biol. Chem. **286:**10276–10287. http://dx.doi.org/10.1074/jbc.M110.200501.
3. **Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F.** 2002. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **46:**2720–2722. http://dx.doi.org/10.1128/AAC.46.8.2720-2722.2002.
4. **Hartkoorn RC, Sala C, Neres J, Pojer F, Magnet S, Mukherjee R, Uplekar S, Boy-Rottger S, Altmann KH, Cole ST.** 2012. Towards a new tuberculosis drug: pyridomycin—nature's isoniazid. EMBO Mol. Med. **4:**1032–1042. http://dx.doi.org/10.1002/emmm.201201689.
5. **Milano A, Pasca MR, Provvedi R, Lucarelli AP, Manina G, Ribeiro AL, Manganelli R, Riccardi G.** 2009. Azole resistance in Mycobacterium tuberculosis is mediated by the MmpS5-MmpL5 efflux system. Tuberculosis (Edinb.) **89:**84–90. http://dx.doi.org/10.1016/j.tube.2008.08.003.
6. **Tekaia F, Gordon SV, Garnier T, Brosch R, Barrell BG, Cole ST.** 1999.

Analysis of the proteome of Mycobacterium tuberculosis in silico. Tuber. Lung Dis. **79:**329–342. http://dx.doi.org/10.1054/tuld.1999.0220.

7. **Domenech P, Reed MB, Barry CE, III.** 2005. Contribution of the Mycobacterium tuberculosis MmpL protein family to virulence and drug resistance. Infect. Immun. **73:**3492–3501. http://dx.doi.org/10.1128/IAI.73.6.3492-3501.2005.
8. **Wells RM, Jones CM, Xi Z, Speer A, Danilchanka O, Doornbos KS, Sun P, Wu F, Tian C, Niederweis M.** 2013. Discovery of a siderophore export system essential for virulence of Mycobacterium tuberculosis. PLoS Pathog. **9:**e1003120. http://dx.doi.org/10.1371/journal.ppat.1003120.
9. **Jones CM, Wells RM, Madduri AV, Renfrow MB, Ratledge C, Moody DB, Niederweis M.** 2014. Self-poisoning of Mycobacterium tuberculosis by interrupting siderophore recycling. Proc. Natl. Acad. Sci. U. S. A. **111:**1945–1950. http://dx.doi.org/10.1073/pnas.1311402111.
10. **Gupta S, Cohen KA, Winglee K, Maiga M, Diarra B, Bishai WR.** 2014. Efflux inhibition with verapamil potentiates bedaquiline in Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **58:**574–576. http://dx.doi.org/10.1128/AAC.01462-13.
11. **Williams K, Minkowski A, Amoabeng O, Peloquin CA, Taylor D, Andries K, Wallis RS, Mdluli KE, Nuermberger EL.** 2012. Sterilizing activities of novel combinations lacking first- and second-line drugs in a murine model of tuberculosis. Antimicrob. Agents Chemother. **56:**3114–3120. http://dx.doi.org/10.1128/AAC.00384-12.
12. **Makarov V, Lechartier B, Zhang M, Neres J, van der Sar AM, Raadsen SA, Hartkoorn RC, Ryabova OB, Vocat A, Decosterd LA, Widmer N, Buclin T, Bitter W, Andries K, Pojer F, Dyson PJ, Cole ST.** 5 February 2014. Towards a new combination therapy for tuberculosis with next generation benzothiazinones. EMBO Mol. Med. http://dx.doi.org/10.1002/emmm.201303575.
13. **Lechartier B, Hartkoorn RC, Cole ST.** 2012. In vitro combination studies of benzothiazinone lead compound BTZ043 against Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **56:**5790–5793. http://dx.doi.org/10.1128/AAC.01476-12.
14. **Diacon AH, Donald PR, Pym A, Grobusch M, Patientia RF, Mahanyele R, Bantubani N, Narasimooloo R, De Marez T, van Heeswijk R, Lounis N, Meyvisch P, Andries K, McNeeley DF.** 2012. Randomized pilot trial of eight weeks of bedaquiline (TMC207) treatment for multidrug-resistant tuberculosis: long-term outcome, tolerability, and effect on emergence of drug resistance. Antimicrob. Agents Chemother. **56:**3271–3276. http://dx.doi.org/10.1128/AAC.06126-11.
15. **Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V.** 2005. A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. Science **307:**223–227. http://dx.doi.org/10.1126/science.1106753.
16. **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:**1022–1028. http://dx.doi.org/10.1128/AAC.01611-09.