Role of Alanine Racemase Mutations in Mycobacterium tuberculosis D-Cycloserine Resistance

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ABSTRACT A screening of more than 1,500 drug-resistant strains of Mycobacterium tuberculosis revealed evolutionary patterns characteristic of positive selection for three alanine racemase (Alr) mutations. We investigated these mutations using molecular modeling, in vitro MIC testing, as well as direct measurements of enzymatic activity, which demonstrated that these mutations likely confer resistance to D-cycloserine.

KEYWORDS Mycobacterium tuberculosis, cycloserine, alanine racemase

In 2015, the Global Drug Facility declared that the cost of D-cycloserine (DCS), a group C drug to treat tuberculosis (TB), would be cut by more than half to as little as $0.19 per capsule to support the treatment of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, which represent a major threat to public health (1). In light of this announcement, a better understanding of the resistance mechanisms to this drug is required to facilitate phenotypic as well as genotypic drug susceptibility testing (DST), both in the context of surveillance and individual patient treatment, to avoid the severe side effects of this drug (2, 3).

Studies of the mode of action of DCS in mycobacteria have produced contradictory results, with some studies pointing to alanine racemase (Alr) as the primary target and others supporting D-alanine–D-alanine ligase (DdlA) (4–9). However, molecular data...
from *Mycobacterium tuberculosis* complex (MTBC) have implicated only *alr* in DCS resistance, which can also be conferred by mutations in alanine dehydrogenase (*ald*) or a permease (*cycA*) gene (10, 11). Using molecular modeling, we had predicted that the *alr* M319T mutation observed in an XDR strain would likely confer resistance to DCS, which was subsequently confirmed by Desjardins et al. using the unrelated strain TKK_04_0105 (see Table S1 in the supplemental material) (2, 11). Desjardins et al. described a number of additional *alr* mutations in strains with elevated DCS MICs, including a C-to-T nucleotide change 8 bp upstream of the experimentally confirmed start codon of *alr* (strain TKK_02_0050 in Table S1) (11, 12). This was notable, as Merker et al. had previously reported that, compared with the susceptible parental *alr* wild-type strain, the acquisition of this mutation during treatment with DCS correlated with DCS resistance, which suggested that *alr* mutations might be both necessary and sufficient to confer DCS resistance (13).

To gain further insights into the impact of *alr* mutations, we first confirmed that the aforementioned *alr* C-8T promoter mutant that evolved during treatment correlated in MICs above the current World Health Organization (WHO)-endorsed critical concentration (CC) of 30 μg/ml using the 1% proportion method on Löwenstein-Jensen (LJ) (strains PBm0 and PBm14 in Table S1; Desjardins et al. and Merker et al. had used 10% as the critical proportion and therefore had not adhered to the current WHO recommendations [11, 13, 14]). Using the same method, we also showed that two strains with *alr* M319T or Y364D mutations from XDR TB patients with a treatment history with DCS had MICs above the CC (Table S1). Moreover, we observed the M319T mutation in three XDR strains (PT1, PT2, and PT5) from Lisbon, Portugal (15). Although no CC exists for MGIT 960, this mutation correlated in an MIC increase from 16 to 64 μg/ml compared with three closely related wild-type control strains (PT3, PT6, and PT7) and one more distantly related control strain (PT4), which supported the role of this mutation in DCS resistance (Fig. 1A; Table S1). In contrast, no or minimal MIC increases were recorded when testing these Portuguese strains using Sensititre MycoTB plates (Table S1) (16).

Finally, a pre-XDR *alr* R373L mutant from a patient with DCS exposure, which also harbored a deletion in *ald*, tested resistant on LJ using the 1% proportion method (Tables S1 and S2).

To study the importance of the C-8T, M319T, Y364D, and R373L mutations from an evolutionary perspective, we screened previously published and unpublished genomes of more than 1,500 MDR strains (mostly from Germany, eastern Europe, and Swaziland), which identified eight additional strains with mutations at these *alr* positions or codons (Table S1). Interrogating the genomes of these 17 strains in the context of a phylogenetically diverse reference collection that included all major MTBC lineages and species showed that the mutations had either been acquired multiple times independently and/or that different amino acid changes were present at the same codons (Fig. 1B). These mutation patterns are typically a signal of positive selection, which could have occurred in response to DCS exposure.

Molecular modeling of these coding mutations supported this hypothesis. *Alr* functions as a homodimer, aided by the cofactor pyridoxal 5′-phosphate (PLP) to which it is covalently bound. DCS inhibits Alr irreversibly by covalently bonding to PLP (4). We generated and analyzed a model of the complex between the *M. tuberculosis* Alr and DCS (Alr\_mtb-DCS) (Fig. S1) (4, 17). Amino acid residues 319 and 364 were located directly in the active site (Fig. S1B). M319T was positioned close enough to allow interaction with the DCS moiety, which, given the large change of the character of the side chain, could strongly affect DCS reactivity (Fig. S1C). Y364 is involved in the positioning of the phosphate moiety of PLP and thus represents a prominent active site residue in the conserved inner layer of the substrate entrance corridor of Alr (Fig. S1B) (17). A mutation to aspartic acid introduced a shorter and negatively charged side chain, which could potentially affect PLP orientation in the active site (Fig. S1C). Moreover, it could influence DCS uptake through alteration of the entrance corridor. Interestingly, M319 is located near Y364 and, as a result, it is possible that the M319T mutation could alter the interaction with Y364, thereby affecting DCS inhibition. In contrast, the R373L mutation
was not directly located within the active site but near the dimer interface and close to residues M319 and D320, which play an important role in the makeup of the active site (Fig. S1B). Consequently, the replacement of arginine with the short and hydrophobic side chain of leucine might disrupt molecular interactions at the dimer interface as well as destabilize the DCS binding site (Fig. S1C).
To test these predictions experimentally, we expressed and purified the aforementioned Alr<sub>Mtb</sub>-coding mutants, along with wild-type Alr<sub>Mtb</sub>, and determined their half-maximal inhibitory concentration (IC<sub>50</sub>) to measure the effectiveness of inhibition by DCS (Fig. 2). The IC<sub>50</sub> for wild-type Alr<sub>Mtb</sub> was 26.4 ± 1.7 μM, which was in the range previously reported for this compound (18, 19). From our structure-based analysis, we expected the two mutations located in the active site to show the greatest effect on DCS inhibition. Indeed, the M319T mutant enzyme showed minimal inhibition by DCS, even at 1,000 μM (Fig. 2). Thus, the IC<sub>50</sub> of this mutant could not be determined. The IC<sub>50</sub> of the Y364D mutant showed a 50-fold increase to 1,328.0 ± 340.0 μM. The R373L mutation, which was not located directly within the active site, also showed a significant increase in resistance to DCS, with an IC<sub>50</sub> of 712.0 ± 138.5 μM (27-fold increase).

Taken together, these data suggested that alr mutations likely confer DCS resistance, although allelic exchange experiments are required to formally prove this (particularly for R373L, which coincided with a deletion in ald and, consequently, may not be sufficient to confer resistance on its own). Although the relationship between MICs and IC<sub>50</sub>s can be complex, the observation that MICs increased by only 4- to 16-fold versus at least 25-fold increases for IC<sub>50</sub>s supports the notion that DCS inhibits multiple targets, as noted earlier. This study should be complemented with extensive MIC testing of phylogenetically diverse pansusceptible MTBC strains to define the epidemiological cutoff value, given that it is unclear based on which evidence the current WHO CC on LJ has been set (3, 14, 20, 21). Moreover, further MIC testing of likely DCS-resistant strains is needed to investigate whether the Sensititre system is less reliable at detecting DCS resistance than are LJ and MGIT. Finally, the impact of alr mutations on resistance on terizidone remains to be investigated.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01575-17.

SUPPLEMENTAL FILE 1, PDF file, 3.3 MB.

ACKNOWLEDGMENTS

This work was funded by the University of Otago, Health Research Council Explorer grant and Maurice Wilkins Centre. In addition, parts of this study were supported by the European Union PathoNgenTrace project (grant FP7-278864-2) and the German Center for Infection Research (DZIF). Further funds were received from Fundação para a Ciência e a Tecnologia, Portugal, through the grants UID/Multi/04413/2013 (to M.V. and D.M.), SFRH/BDP/100688/2014 (to D.M.), and SFRH/BDP/95406/2013 (to J.P.). F.C. was supported by the Wellcome Trust 201344/Z/16/Z. T.G.C. was funded by the Medical Research Council UK (grants MR/K000551/1, MR/M01360X/1, and MR/N010469/1). Fur-
ther support was received the Indian Council of Medical Research, New Delhi and Health Innovation Challenge Fund (grants HICF-T5-342 and WT098600), a parallel funding partnership between the UK Department of Health and the Wellcome Trust. C.U.K. is a research associate at Wolfson College, Cambridge, UK.

The views expressed in this publication are those of the authors and not necessarily those of the Department of Health, Public Health England, or the Wellcome Trust.

K.L.K., Y.N., and H.K.O.-R. have received funding for alanine racemase-related projects from L2 Diagnostics LLC, New Haven, CT. J.P., S.J.P., and C.U.K. have collaborated with Illumina, Inc. on a number of scientific projects. J.P. has received funding for travel and accommodation from Pacific Biosciences, Inc. and Illumina, Inc. S.J.P. has received funding for travel and accommodation from Illumina, Inc. C.U.K. is a consultant for the Foundation for Innovative New Diagnostics. The Bill & Melinda Gates Foundation and Janssen Pharmaceutical covered C.U.K.’s travel and accommodation to present at meetings. The European Society of Mycobacteriology awarded C.U.K. and M.M. the Gertrud Meissner Award, which is sponsored by Hain Lifescience.

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