Respiratory Flexibility in Response to Inhibition of Cytochrome c Oxidase in *Mycobacterium tuberculosis*

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We report here a series of five chemically diverse scaffolds that have *in vitro* activities on replicating and hypoxic nonreplicating bacilli by targeting the respiratory bc_1 complex in *Mycobacterium tuberculosis* in a strain-dependent manner. Deletion of the cytochrome *bd* oxidase generated a hypersusceptible mutant in which resistance was acquired by a mutation in *qcrB*. These results highlight the promiscuity of the bc_1 complex and the risk of targeting energy metabolism with new drugs.

Tuberculosis remains a leading cause of death despite more than half a century of chemotherapeutic intervention. Bedaquiline (BDQ), the first FDA-approved antitubercular in more than 4 decades, was recently accepted by the U.S. FDA for the treatment of multidrug- and extensively drug-resistant disease (1). This diarylquinoline drug is thought to target the *Mycobacterium tuberculosis* ATP synthase, a critical component of mycobacterial energy metabolism (2). Recently, a new clinical candidate based on an imidazo[1,2-α]pyridine scaffold was disclosed (3). We previously described this scaffold from a screening effort specifically designed to identify inhibitors of respiratory enzymes, and it subsequently appeared from several other screening efforts (4–6). Mutants resistant to this series were mapped to an amino acid mutation in the QcrB subunit of the menaquinol cytochrome *c* oxidoreductase (bc_1 complex), which is part of the bc_1-aa_3-type cytochrome *c* oxidase complex (3, 5).

We previously established that the majority of clinical strains of *M. tuberculosis* tested were susceptible to full growth inhibition by the imidazo[1,2-α]pyridines (4, 7, 8) but found that the laboratory-adapted strains H37Rv, CDC1551, and Erdman overcame this growth inhibition, despite the ability of these respiratory inhibitors to block the transfer of electrons to resazurin (Tables 1

and 2, compound 2; Fig. 1). The outgrowth observed with the laboratory-adapted strains was not due to the acquisition of resistance, since the cells showed the same phenomenon of outgrowth upon subculturing in drug-free medium followed by repeated MIC testing. We hypothesized that the ability of *M. tuberculosis* H37Rv to overcome bc_1 complex inhibition was due to the upregulation of the cytochrome *bd* oxidase and *cydDC* genes (4), an alternative respiratory complex. Indeed, standing cultures that mimic the less oxygenated physiology of cells in MIC assays of the laboratory-adapted strains had higher basal expression levels of the *cydA* gene than did sensitive clinical strains (see Table

TABLE 1 *In vitro* evaluation of compounds that target the bc_1 complex

Strain	*qcrB* mutation	1 MIC[a]	1 MABA[b]	2 MIC	2 MABA	3 MIC	3 MABA	4 MIC	4 MABA	5 MIC	5 MABA
K14b0DS	WT[c]	0.6	0.39	0.01	0.024	4.7	1.56	4.7	2.3	25	4.7
H37Rv[d]	WT	>50	2.3	>50	0.15	>50	12.5	37	4.7	>50	50
cydKO	WT	0.4	0.4	<0.024	<0.024	2.3	2.3	3.1	2.3	4.7	3.1

[a] MIC values are in μM.
[b] Microplate alamarBlue assay (MABA) results (the concentration of compound that inhibits resazurin reduction by >90%) are in μM.
[c] WT, wild type.
[d] Similar results were seen with the laboratory strains CDC1551 and Erdman.

TABLE 2 Resistance to compounds that target the bc_1 complex

Protein	$qcrB$ mutation	Fold resistance to compound:				
		1	2	3	4	5
Qcr1	A317V	>128	940.0	>22	16.0	>11
Qcr2	M342T	24.1	78.0	8.3	8.0	1.3
Qcr3	W312G	94.9	313.0	>22	8.0	>11
Qcr4	A396T	5.9	7.0	16.1	6.1	5.3
Qcr5	M342I	32.1	30.0	>22	3.0	0.3
Qcr6	A317T	8.0	60.0	21.7	11.8	>11
Qcr7	S182P	48.7	20.0	>22	8.0	>11

S1 in the supplemental material). Although the laboratory and clinical K14b0DS strains upregulated *cydA* in response to QcrB inhibition, the final levels of cytochrome *bd* oxidase in the clinical strain did not match those evident in the laboratory-adapted strain (see Fig. S1 in the supplemental material). (Strain K14b0DS was collected with NIAID institutional review board approval under protocol [ClinicalTrials.gov identification no. NCT00341601].)

The ability of laboratory-adapted strains to overcome bc_1 complex inhibition by upregulation of the cytochrome *bd* oxidase led us to speculate that a knockout mutant of this complex in the laboratory strain H37Rv would remain susceptible to such inhibitors. We deleted this oxidase in H37Rv by replacing a 221-bp MluI fragment in the *cydABDC* operon with the *aph* gene encoding an aminoglycoside phosphotransferase. The mutant lacks the 3' end of the *cydB* gene (encoding subunit II of the cytochrome *bd* oxidase), the entire *cydD* gene, and the 5' end of *cydC* (where *cydDC* encodes a transporter involved in cytochrome biogenesis). The cytochrome *bd* oxidase mutant (cydKO) was found to be highly susceptible to the imidazo[1,2-α]pyridines (Table 1). To map the binding site of the inhibitors in the bc_1 complex, we generated mutants resistant to compound 1 in the cydKO strain. Mutants were raised at a frequency of 10^{-8}, and sequencing of the *qcrB* gene revealed single-nucleotide polymorphisms (SNPs) leading to 7 different amino acid mutations that all mapped to the stigmatellin binding site of the bc_1 complex (9) (Table 2).

We identified four other diverse chemotypes that exhibited the same time-dependent inability to maintain growth inhibition of *M. tuberculosis* H37Rv while maintaining the ability to inhibit resazurin reduction (Table 1) and rapidly inhibit ATP production with kinetics similar to those of BDQ and the protonophore carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) (see Fig. S2 in the supplemental material). The cydKO mutant was hypersusceptible to these compounds, whereas the *qcrB* mutants in the genetic cydKO background were resistant to them; mutations at Met342 did not confer resistance to compound 5, suggesting that this compound does not interact with this residue. Compound 1 is an imidazo[4,5-*c*]pyridine that was previously reported to have submicromolar potency against *M. tuberculosis* (10). The ability of all these scaffolds to inhibit the mycobacterial bc_1 complex raised the concern that they might similarly inhibit mitochondrial respiration, which can lead to deleterious side effects during chemotherapy *in vivo*. Therefore, we conducted cytotoxicity testing against HepG2 cells using an assay which forces cells to use mitochondrial respiration by growth in galactose (11). It was found that compounds 3, 4, and 5 inhibited mitochondrial respiration (see Table S2 in the supplemental material), further reinforcing the notion that these compounds affect respiratory cytochrome function. A homology model of QcrB was constructed with multiple cytochrome *b* homologues (PDB no. 3CX5 [yeast], 1NTM [bovine], 1ZRT [*Rhodobacter capsulatus*], 2QJP [*Rhodobacter sphaeroides*], and 3H1H [chicken]) using MP-T (12) and MODELLER (13). The mutated residues were found to lie in close proximity in the pocket that binds stigmatellin, a known inhibitor in other species, suggesting a mechanism analogous to stigmatellin involving disruption of electron transport to the iron-sulfur protein. Although part of the pocket lies in a region of significant divergence in *M. tuberculosis*, it was nevertheless possible to dock the compounds in a plausible binding mode consistent with the mutational results using the software GOLD (14), allowing an elastic distance potential to the mutant residues (Fig. 2).

The discovery of five distinct scaffolds that all target the *M. tuberculosis* bc_1 complex suggests that this membrane target is promiscuous, as reported for several other membrane-associated targets (15). The hypersusceptibility of the cydKO mutant, in combination with the discordance between growth inhibition and resazurin reduction, offers a means of identifying such inhibitors early in the drug discovery process.

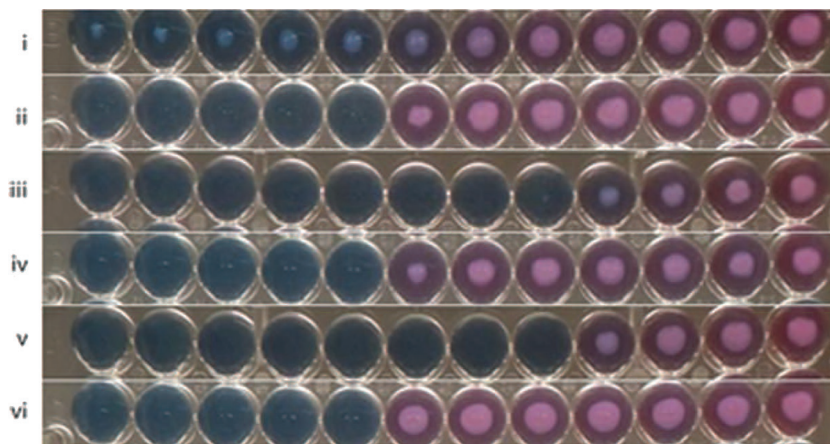Since the discovery of BDQ, there has been significant enthu-

FIG 1 Treatment of *M. tuberculosis* isolates with inhibitors of the bc_1 complex results in inhibition of resazurin reduction. *M. tuberculosis* isolates were treated with 2-fold serial dilutions of compound 2 (lanes i, iii, and v) or isoniazid (lanes ii, iv, and vi) for 2 weeks followed by addition of alamarBlue. Lanes i and ii, H37Rv; lanes iii and iv, K14b0DS; lanes v and vi, cydKO.

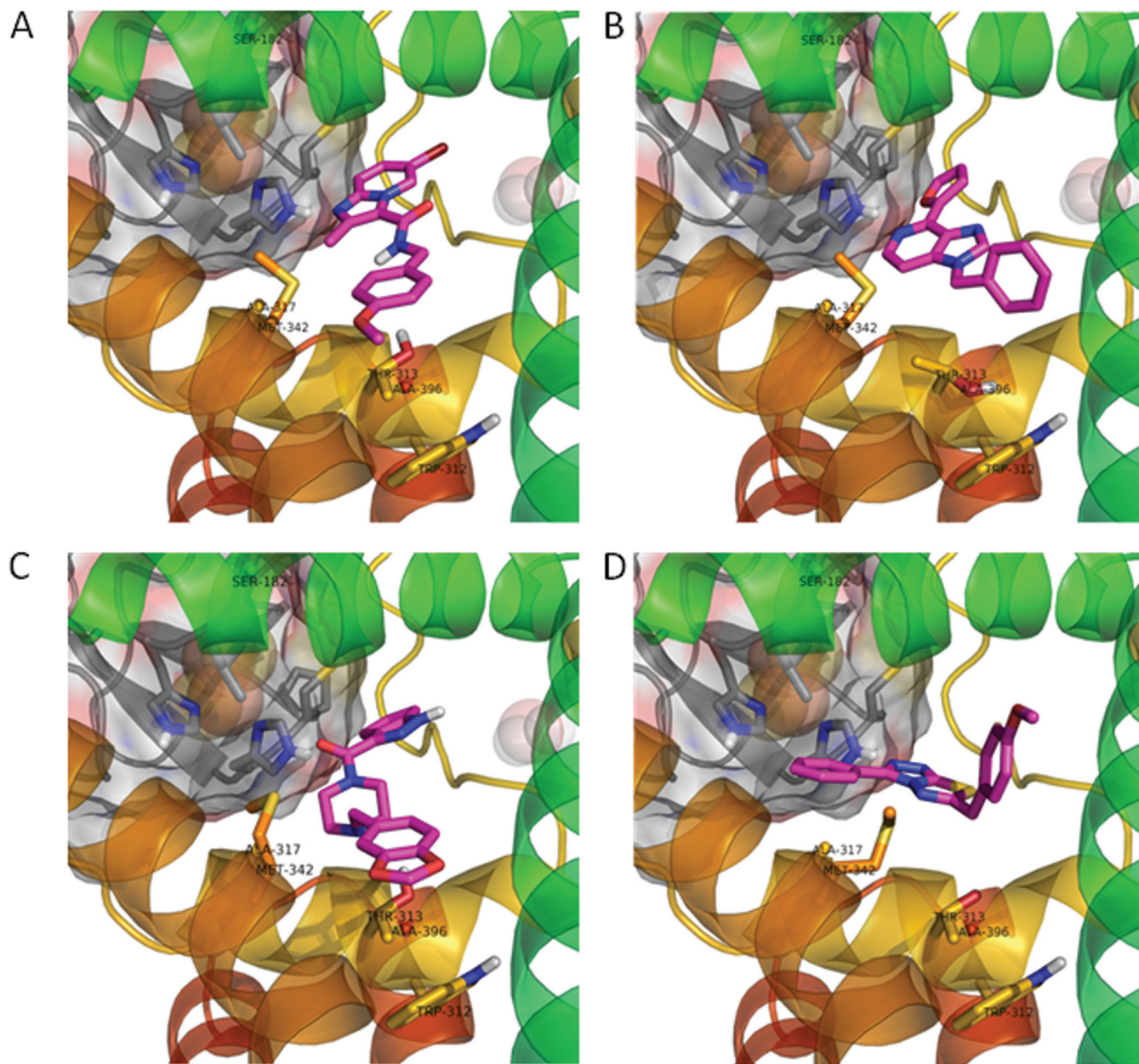**FIG 2** Modeling of the imidazopyridine binding pocket of QcrB showing docking poses for the two scaffolds. (A) Docking of imidazo[1,2-α]pyridine (compound 2) in the QcrB homology model; (B) docking of imidazo[4,5-c]pyridine (compound 1) in QcrB; (C) docking of compound 3; (D) docking of compound 4. Compounds are shown in magenta, iron-sulfur protein in gray, and QcrB in rainbow spectrum; resistance-conferring residues are labeled and shown in stick representation.

siasm for new targets that might disrupt energy metabolism, which is now perceived as a metabolic vulnerability in nonreplicating *M. tuberculosis* (16). Our results suggest that the pathways involved in respiration and energy metabolism show significant metabolic plasticity and can be altered during long-term laboratory adaption of clinical isolates. Laboratory passage of *M. tuberculosis* strains has likely resulted in many adaptations to *in vitro* growth over time (17). In this instance, the tight control of the *cydAB* genes in strain H37Rv seems to have been lost, allowing a buffered response to the reduced functioning of the *bc*₁ complex. Significantly, our results show that *M. tuberculosis* has the respiratory flexibility to not only survive but also actually grow using

the alternative cytochrome *bd* oxidase complex during chemical inhibition of the *bc*₁ complex, consistent with observations in *Mycobacterium smegmatis* (18). Our understanding of the regulation of the respiratory network in *M. tuberculosis* is minimal, although it is known that the cytochrome *bd* oxidase complex is upregulated under hypoxic conditions (19). These results argue for caution when assuming that an effect observed on aerobic organisms (such as those found in sputum) will translate into an effect on microaerophilic organisms (such as those found in lesions) and further highlight the risk of targeting respiration in drug development without understanding the relative contribution of the respiratory complexes in human granuloma.

ACKNOWLEDGMENTS

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