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**Naturally-occurring polymorphisms in QcrB are responsible for resistance to
Telacebec in *Mycobacterium abscessus***

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Mycobacterium abscessus is a rapidly growing non-tuberculous mycobacteria that is quickly emerging as a global health concern. *M. abscessus* pulmonary infections are frequently intractable due to the high intrinsic resistance to most antibiotics. Therefore, there is an urgent need to discover effective pharmacological options for *M. abscessus* infections. In this study, the potency of the anti-tuberculosis drug Telacebec (Q203) was evaluated against *M. abscessus*. Q203 is a clinical-stage drug candidate targeting the sub-unit QcrB of the cytochrome *bc₁:aa₃* terminal oxidase. We demonstrated that the presence of four naturally-occurring polymorphisms in the *M. abscessus* QcrB are responsible for the high resistance of the bacterium to Q203. Genetics reversion of the four polymorphism sensitized *M. abscessus* to Q203. While this study highlights the limitation of a direct drug repurposing approach of Q203 and related drugs for *M. abscessus* infections, it reveals that the *M. abscessus* cytochrome *bc₁:aa₃* respiratory branch is sensitive to chemical inhibition.

Keywords: Q203, cytochrome *bc₁:aa₃*, NTM, bedaquiline, antimicrobial resistance, cytochrome *bd* oxidase, tuberculosis.

The global incidence of rapidly growing mycobacteria (RGM) have increased at an alarming rate, particularly in developed countries.¹⁻³ Chief among them is the *Mycobacterium abscessus* (Mabs) complex that accounts for the majority of RGM-related infections.⁴⁻⁵ The Mabs complex is comprised of three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*.⁶ Mabs primarily causes pulmonary infections among the elderly and individuals with pre-existing lung pathology, such as cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary diseases (COPD).⁷⁻⁹ Mabs pulmonary infections are notoriously difficult to treat as the pathogen is equipped with intrinsic mechanisms that mediate resistance to classical anti-tuberculous (anti-TB) drugs and most clinically available antimicrobial agents.¹⁰⁻¹² Despite the long treatment duration of up to 12 months, the current recommended treatment regimen consisting of a combination of amikacin, clarithromycin, and imipenem is unreliable, with failures and relapses reported in almost 70% of the patients.¹³⁻¹⁵ Hence, new and effective therapeutic options are urgently needed.

In recent years, the oxidative phosphorylation (OxPhos) pathway has emerged as an attractive target space for drug development against *Mycobacterium tuberculosis* (Mtb).¹⁶⁻¹⁹ Interestingly, while most anti-TB drugs are ineffective against Mabs, those targeting the OxPhos pathway, such as Bedaquiline and Clofazimine, retained some potency.²⁰⁻²² Telacebec (Q203) is a drug candidate in Phase 2 clinical trial that targets the cytochrome *bc₁:aa₃* (Cyt-*bc₁:aa₃*), one of two terminal oxidases expressed by most mycobacteria.^{18, 23-24} However, the potency and efficacy of Q203 has not been evaluated in Mabs.

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In this study, we found that Q203 is ineffective against laboratory-adapted and clinical strains of *Mabs* complex. Resistance is caused by naturally-occurring genetic polymorphisms in the target gene of Q203, namely the QcrB subunit of the Cyt-*bc₁:aa₃* complex.

RESULTS AND DISCUSSION

Q203 is inactive in *M. abscessus*

Given the promise of targeting the Cyt *bc₁-aa₃* in *Mtb*, we evaluated the potency of Q203, a potent inhibitor of the *Mtb* Cyt *bc₁-aa₃* against *Mabs* strain CIP104536^T.²⁵ We observed that Q203 failed to exhibit growth inhibitory activity up to a concentration of 25 μM (Figure 1A), a concentration approximately 10,000-fold of its Minimum Inhibitory Concentration (MIC₅₀) in *Mtb*.¹⁸ Lack of potency was also observed in seventeen clinical isolates, showing that resistance to Q203 is not restricted to the laboratory-adapted *Mabs* strain CIP104536^T strain (Table 1). As a control, we showed that all the strains were sensitive to the F_oF₁ ATP synthase inhibitor, Bedaquiline (BDQ) (Figure 1A, Table 1A).^{17, 20}

The presence of the cytochrome-*bd* oxidase (Cyt-*bd*), an alternate terminal oxidase, is known to interfere with the efficacy of Q203 in *Mtb*.²⁶ The extent of the synthetic lethal interaction between the Cyt-*bc₁:aa₃* and the Cyt-*bd* in *Mabs* has yet to be determined. To determine if the inactivity of Q203 in *Mabs* was due to the presence of the Cyt-*bd*, we tested the potency of Q203 against a *Mabs* CIP104536^T Δ*cydABDC* strain constructed in the context of this work (Supplementary Figures 1, 2). Q203 was similarly inactive in the *Mabs* CIP104536^T Δ*cydABDC* strain (Figure 1B), indicating that the compensatory action of the Cyt-*bd* oxidase is not responsible for lack of potency of Q203 in *Mabs*.

Figure 1. Q203 is inactive in *M. abscessus*. Dose-response curves of Q203 (blue circles) and Bedaquiline (BDQ) (red squares) against Mabs CIP104536^T (A) parental and (B) Δ cydABDC strains. The OD₆₀₀ values were measured after 48 hours of incubation at 32°C in the presence of a dose-range of the drugs. Data are expressed as the mean $\pm$ SDs of triplicates for each concentration.

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Table 1. MIC₅₀ values of Q203 and BDQ against clinical isolates of *M. abscessus* complex^a.

Isolates ref No.	Subspecies	Morphotype	Q203	BDQ
			MIC ₅₀ (μM)	MIC ₅₀ (μM)
17	<i>M. abscessus</i>	R	>50	0.68
18	<i>M. abscessus</i>	S	>50	0.13
19	<i>M. abscessus</i>	R	>50	0.11
20	<i>M. abscessus</i>	S	>50	0.07
21	<i>M. abscessus</i>	S	>50	0.14
22	<i>M. abscessus</i>	R	>50	0.05
23	<i>M. abscessus</i>	R	>50	0.12
24	<i>M. abscessus</i>	R	>50	0.07
25	<i>M. abscessus</i>	S	>50	0.03
26	<i>M. abscessus</i>	S	>50	0.37
27	<i>M. massiliense</i>	R	>50	0.09
28	<i>M. massiliense</i>	R	>50	0.04
29	<i>M. massiliense</i>	R	>50	0.10
31	<i>M. massiliense</i>	R	>50	0.10
33	<i>M. massiliense</i>	S	>50	0.08
34	<i>M. massiliense</i>	S	>50	0.05
35	<i>M. abscessus</i>	R	>50	0.10

^aThe MIC₅₀ values indicate the minimum drug concentration at which 50% of bacterial growth was inhibited in a full dose-response curve. BDQ: Bedaquiline. R: rough; S: smooth.

Presence of four single-nucleotide polymorphisms associated with resistance to Q203 in Mabs *QcrB*

The *QcrB* subunit of the cytochrome *bc1* complex is the binding target of Q203.¹⁸ Alignment of the *QcrB* sequences between Mabs strain CIP104536^T and *Mtb* strain H37Rv revealed four amino acid polymorphisms for which substitutions have been associated with resistance to *QcrB* inhibitors in *Mtb*.²⁷ Based on the *Mtb* sequence, the four amino acids are S182G; E314D; A317L; A396C (Figure 2). A TBLASTN search against all published *M. abscessus* genomes available at NCBI database, using the *QcrB* sequence (YP_001702702.1) of the *M. abscessus* strain CIP104536^T as the query sequence, revealed that these polymorphisms are present in all 1,662 *M. abscessus* isolates sequenced to date. Of the 1,662 isolates, 838 are *M. abscessus subsp. abscessus*, 351 *M. abscessus subsp. Massiliense*, and 115 *M. abscessus subsp. Bolletii* subtypes. The subtypes of the remaining 358 isolates are not defined. Therefore, we hypothesized that these substitutions are the cause of Mabs resistance to Q203 across the vast majority of circulating Mabs clinical isolates.

Mtb_QcrB	SSITLGMPIVIGTWLHWALFGGDFPGTILIPRLYALHILLPGIILALIGLHALVWFQKH	240
Mabs_QcrB	SGITMGLPLIGTWMMHWALFGGDFPGNIIIPRLYAMHILLIPAIILALIGIHLALVWYQKH	237
	*.:**::*:*****:*****.*****:*****:*.*****:*****:***	
Mtb_QcrB	TQFPGPGRTEHNVVGVVRMPVFAFKSGAFFAAIVGVLGLMGGLLQINPIWNLGPYKPSQV	300
Mabs_QcrB	TQFPGPGATEKNVVGVRILPVFALKGGSFFAFTTAILALMSGLLQINPIWVLGPYKPSQI	297
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Mtb_QcrB	SAGSQPDFYMMWTEGLARIWPPWEFYFWHHTIPAPVWVAVIMGLVFVLLPAYPFLEKRFT	360
Mabs_QcrB	SAGSQPDFYMMWTDGLLRIIPAWEIYPFGHTIPQAVWVAVGMGLVFGLLIAYPFLEKKLT	357
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Mtb_QcrB	GDYAHNNLLQRPDRVPRTAIGAMAIAYFMYLTLTAMNDIIALKFHISLNATTWIGRIGM	420
Mabs_QcrB	GDDAHNNLLQRPDAPVRTAIGSAAISLYMLFTLMCMNDIIALKFHISLNATTWIGRIGM	417
	** ***** ***** . **.....** *****	

Figure 2. Alignment of the QcrB sequences between *M. tuberculosis* and *M. abscessus*. The QcrB sequences (amino acids 181 to 420) from Mtb H37Rv (Mtb_QcrB) and Mabs CIP104356^T (Mabs_QcrB) were aligned using the Clustal Omega program. The residues for which substitution are reported to be associated with resistance to Q203 in Mtb are highlighted in red. The four polymorphisms at these positions in the Mabs sequence are highlighted in green/ blue.

Q203 inactivity in *M. abscessus* is due to single nucleotide polymorphisms in QcrB

To validate our hypothesis, we first expressed the Mabs *qcrCAB* operon in *Mycobacterium bovis* BCG, a species susceptible to Q203.²⁶ Upon expression of the Mabs *qcrCAB* operon on a single copy, the recombinant strain became resistant to Q203 (Figure 3A, Supplementary Table 1). The MIC₅₀ of Q203 shifted from 2.7 nM in the parental strain to >100 nM in the recombinant strain. Next, we tested the direct implication of the four Mabs QcrB amino acids hypothesized to confer resistance to Q203 in Mabs. The four amino acids were mutated to their corresponding amino acids in Mtb, giving rise to the chimeric operon Mabs *qcrCAB**. Remarkably, the recombinant *M. bovis* BCG strain transformed with the Mabs *qcrCAB** operon remained susceptible to Q203 (Figure 3A, Supplementary Table 1). As a control, we have shown that the three strains are equally sensitive to bedaquiline (Supplementary Table 1). These results are consistent with the four QcrB amino acids being responsible for the resistance to Q203 in Mabs.

To exclude any artefact related to the use of a surrogate host, we devised a strategy to study the involvement of the natural polymorphisms to the resistance to Q203 in Mabs. The *qcrCAB* operon was deleted in Mabs strain CIP104536^T via homologous recombination to obtain a Mabs strain deficient for the expression of Cyt *bc₁-aa₃* (Δ *qcrCAB* strain) (Supplementary Figure 3). The strain was viable but had a significant growth defect that could be complemented by expressing a parental copy of the deleted genes (Supplementary Figure 4). While the Mabs Δ *qcrCAB* strain complemented with the wild-type Mabs *qcrCAB* operon remained resistant to Q203, expression of the Mabs *qcrCAB** chimeric version in the mutant strain was sufficient to confer susceptibility to Q203 in the low nM range (Figure 3B, Supplementary Table

2). Susceptibility to bedaquiline was unaffected by these genetic manipulations (Supplementary Table 2).

In order to visualize the effect of the four single nucleotide polymorphism on Q203-binding, a three-dimensional model for Mtb QcrB was generated using homology modelling of the *M. smegmatis* EM structure (PDB ID: 6ADQ) as a template.²⁸ Since the sequence identity between Mtb and *M. smegmatis* is 83%, a good model could be predicted with sequence coverage of around 97% and a GMQE (Global Model Quality Estimation) of around 0.90. The four amino acids S182, E314, A317, and A396 in the Mtb QcrB model were substituted by glycine, aspartic acid, leucine and cysteine, respectively, and a pseudo Mabs model was generated. A comparison of both models (r.m.s.d of 0.006 Å) showed a conformational difference for E314 when substituted with aspartate (Supplementary Figure 5A). Moreover, a surface analysis of the Mtb QcrB model showed a cavity that includes the residues S182, E314, A317, A396 (Supplementary Figure 5B), which reveals a few dents near the residues E314 and A317. Interestingly, in the Mabs QcrB model with the substitutions of the residues to glycine, aspartic acid, leucine and cysteine, respectively, the cavity now becomes shallow and the dents are not observed (Supplementary Figure 5C; green arrow). As A396 is buried under the cavity and the side chain of S182 is above the cavity, the substitution of E314D and A317L in Mabs model has a major influence on the cavity variation. Therefore, we speculate that the disparity in the cavity between Mtb and Mabs might affect Q203-binding and thereby explain the loss of potency. A better understanding of the atomistic structural details on how Q203 binds in the proposed cavity and interacts with the four amino acids, S182, E314, A317, and A396 may help rationally designing Mabs QcrB inhibitors in the future.

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3 Taken together, the results demonstrated that Mabs resistance to Q203 is attributable
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5 to natural-occurring polymorphisms found in the drug target, QcrB, rather than other
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7 intrinsic resistance mechanisms such as efflux pumps, cell wall permeability or target-
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9 modifying enzyme. In addition, the study suggest that the development of Cyt *bc₁-aa₃*
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11 inhibitors for Mabs lung diseases can be rationally designed, and possibly be used as
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13 part of a drug combination targeting other components of the OxPhos pathway such
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15 as the Cyt-*bd* terminal oxidase.
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Figure 3. Q203 inactivity in *M. abscessus* is due to single nucleotide polymorphisms in QcrB. (A) Growth inhibitory activity of Q203 against *M. bovis* BCG parental strain (blue circles), BCG complemented with wild-type Mabs *qcrCAB* (red squares) and BCG complemented with chimeric Mabs *qcrCAB* (green triangles). The OD₆₀₀ values were measure after 5 days of incubation at 37°C. (B) Growth inhibitory activity of Q203 against Mabs CIP104536^T Δ *qcrCAB* complemented with the wild-type (blue circles) or chimeric (red squares) Mabs *qcrCAB* operon. The OD₆₀₀ values were measured after 48 hours of incubation at 32°C. Amino acids at positions G179S, D311E, L314A and C393A in Mabs QcrB were mutated to their corresponding amino acids in Mtb to obtain chimeric Mabs *qcrCAB*. Data are expressed as the mean $\pm$ SDs of triplicates for each concentration.

MATERIALS AND METHODS

Strains and growth condition

M. abscessus CIP104536^T and its derivative strains were routinely grown and maintained at 32°C in Middlebrook 7H9 liquid medium supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% ADS supplement. *M. bovis* BCG and its derivative strains were grown and maintained at 37°C in Middlebrook 7H9 liquid medium supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% ADS supplement. Kanamycin (50 µg/mL) or zeocin (20 µg/mL) were used for selection as required. All clinical strains were isolated from the sputum of patients at the time of diagnosis at the Samsung Medical Centre, a 1,979-bed referral hospital in Seoul, South Korea.

Antimicrobial Compounds

Q203 and Bedaquiline (BDQ) were purchased from GVK Biosciences Private Limited, India and Cellagen Technology LLC, USA respectively. All drug stock solution was prepared in 90% dimethyl sulfoxide (DMSO) and stored at -20°C.

Growth Inhibition Assay

M. abscessus CIP104536^T, *M. bovis* BCG and their derivative strains were grown to mid-log phase in Middlebrook 7H9 broth medium supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% ADS supplement, washed twice in Middlebrook 7H9 broth medium supplemented with 0.05% Tween 80, and 10% ADS supplement. Q203 and BDQ dissolved in 90% DMSO were twofold serial-diluted and spotted to 96-well flat bottom plates. 200 µL of the bacteria culture (at OD₆₀₀ 0.005) was inoculated into the 96-well flat bottom and incubated at 32°C (Mabs strains) or 37°C (BCG strains).

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Growth was determined by measuring absorbance at 600 nm (OD₆₀₀) using BioTek Cytation-3 Cell Imaging Multiple-mode reader after 48 hours (Mabs strains) or 5 days (BCG strains) of incubation. MIC₅₀ curves were plotted using the GraphPad Prism 7 software.

Homology modelling of QcrB

The Mtb QcrB model was generated using homology modelling within the SWISS-MODEL server.²⁹ The program accepts the target amino acid sequence and searches for evolutionary related protein structures against the template library SMTL using BLAST and HHblits.³⁰⁻³¹ The templates were then ranked based on global model quality estimate (GMQE) and quaternary structure quality estimate (QSQE). Finally, the 3D model of the Mtb QcrB was generated using the *M. smegmatis* EM structure (PDB ID: 6ADQ) as a template.²⁸ The heme groups were manually modelled in to the Mtb QcrB based on the *M. smegmatis* EM structure (PDB ID: 6ADQ).²⁸ A pseudo model of QcrB for Mabs was generated by substituting the residues S182, E314, A317, A396 in Mtb to glycine, aspartic acid, leucine and cysteine, which has the same score as that of the Mtb model.

ANCILLARY INFORMATION

Supporting information

Supplementary Figure 1: Genetic deletion of *cydABDC* operon in *M. abscessus* CIP104536^T; Supplementary Figure 2: The cytochrome *bd* oxidase is not required for optimum growth in *M. abscessus*; Supplementary Figure 3: Genetic deletion of *qcrCAB* operon in *M. abscessus* CIP104536^T; Supplementary Figure 4: The cytochrome *bc₁:aa₃* is required for optimum growth in *M. abscessus*; Supplementary

Figure 5: Structural model of QcrB from *M. tuberculosis* and pseudo *M. abscessus*;
Supplementary Table 1: MIC₅₀ values of Q203 and bedaquiline (BDQ) calculated from
Figure 3A; Supplementary Table 2: MIC₅₀ values of bedaquiline (BDQ) and Q203
calculated from Figure 3B.

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Notes

The authors declare no competing financial interest

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Abbreviations

Mabs, *Mycobacterium abscessus*; OxPhos, oxidative phosphorylation; Mtb, *Mycobacterium tuberculosis*; Cyt-*bc*₁:*aa*₃, cytochrome *bc*₁:*aa*₃; Cyt-*bd*, cytochrome-*bd* oxidase; BDQ, Bedaquiline.

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