Supplemental Information

Structural and mechanistic study of a novel inhibitor of *M. tuberculosis* cytochrome bc_1:aa_3

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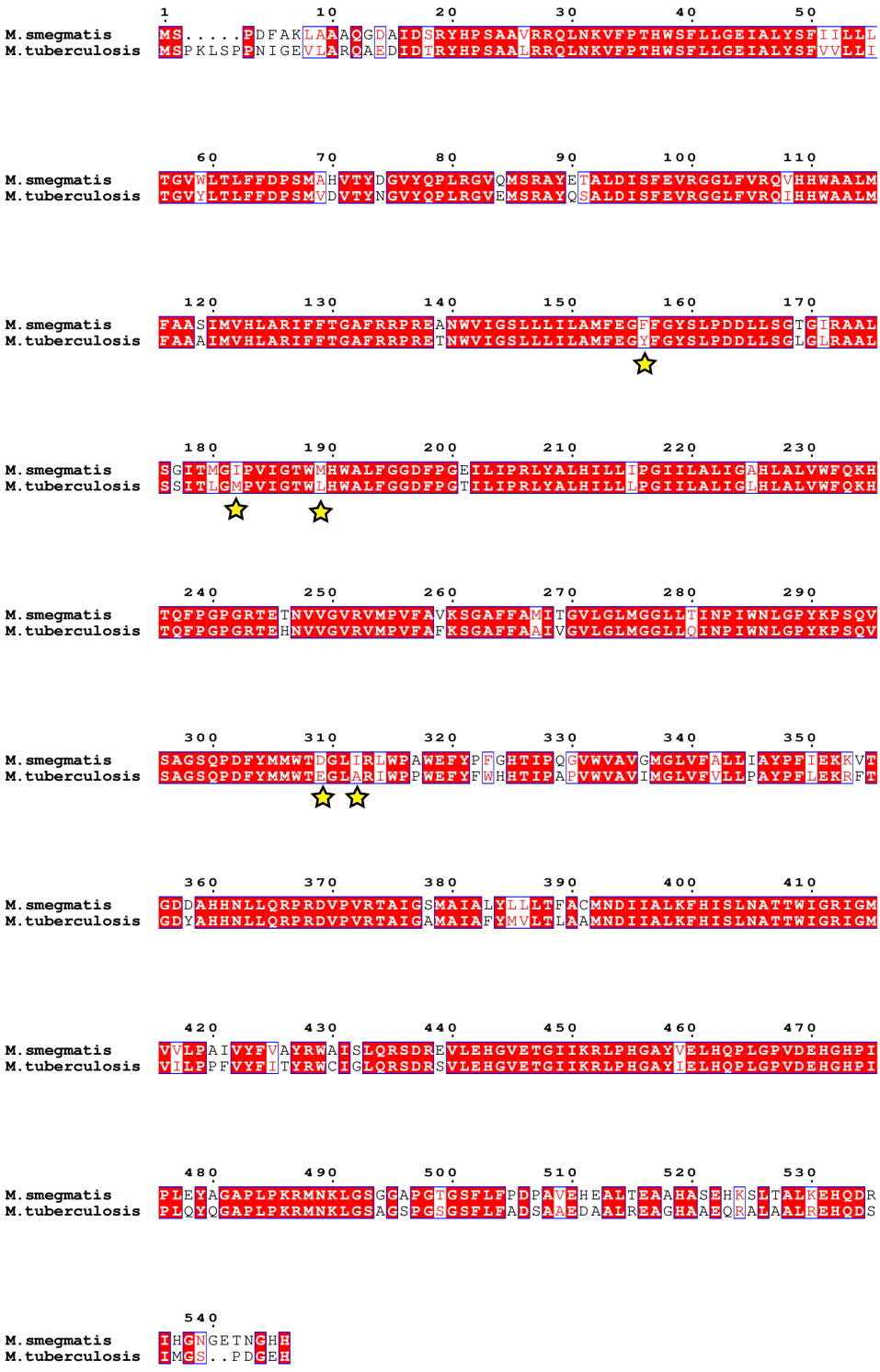
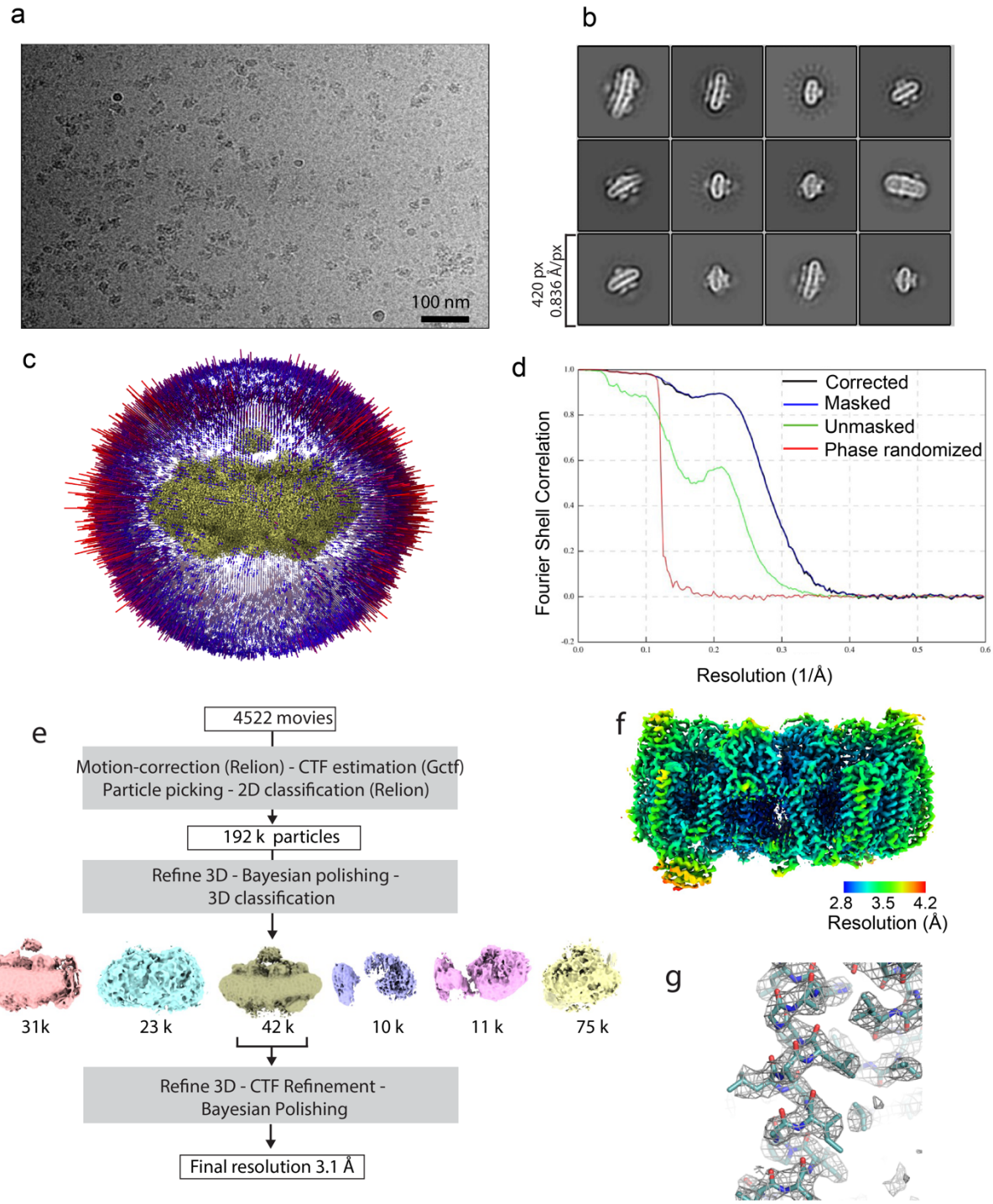
Supplemental Figure 1: Sequence conservation of cytochrome bc QcrA subunit. Top sequence: *M. smegmatis* QcrA, bottom sequence: *M. tuberculosis* QcrA.



Supplemental Figure 2: Sequence conservation of cytochrome bc QcrB subunit. Top sequence: *M. smegmatis* QcrB, bottom sequence: *M. tuberculosis* QcrB. The yellow stars under the sequence mark the five residues in the Qp menaquinol binding site that differ between *M. smegmatis* and *M. tuberculosis*.



Supplemental Figure 3. Structure determination of *M. smegmatis* cytochrome bc$^{Mtb\text{-}like}$. (**a**) cryo-EM micrograph of cytochrome bc. (**b**) Representative 2D classes. (**c**) Orientational distribution of final set of particles (**d**) Fourier Shell Correlation between half-maps at sequential stages of the refinement process. (**e**) Schematic representation of data processing workflow. (**f**) Local resolution map. (**g**) Representative view of the cryo-EM map in grey mesh and fitted model in teal sticks.

Subunit	Mutation	Inhibitor	Reference
QcrB	G175S	CWHM-728	(Harrison-2019)
QcrB	G175S	Quinazoline	(Lupien-2020)
QcrB	L176P	lanzaprazole	(Rybniker-2015)
QcrB	A178T	CWHM-728	(Harrison-2019)
QcrB	A178V	CWHM-728	(Harrison-2019)
QcrB	A179P	PABs	(Chandrasekera-2015)
QcrB	S182P	IPA	(Arora-2014)
QcrB	S182P	AX-35	(Foo-2018)
QcrB	S182P	Q203	(Moraski-2016)
QcrB	W312G	IPA	(Arora-2014)
QcrB	W312G	Q203	(Moraski-2016)
QcrB	W312G	PABs	(Chandrasekera-2015)
QcrB	W312C	PABs	(Chandrasekera-2015)
QcrB	T313I	Q203	(Pethe-2013)
QcrB	T313I	TB47	(Waller-2023)
QcrB	T313I	MOT	(Cleghorn-2018)
QcrB	T313A	Q203	(Pethe-2013)
QcrB	T313A	TB47	(Waller-2023)
QcrB	G315S	CWHM-728	(Harrison-2019)
QcrB	A317V	JNJ-2901	This work
QcrB	A317V	IPA	(Arora-2014)
QcrB	A317V	Q203	(Moraski-2016)
QcrB	A317T	IPA	(Arora-2014)
QcrB	A317T	Q203	(Moraski-2016)
QcrB	A317I	P-P-D[1]	(van der Westhuyzen-2015)
QcrB	V338G	CWHM-728	(Harrison-2019)
QcrB	M342T	JNJ-2901	This work
QcrB	M342T	IPA	(Arora-2014)
QcrB	M342T	Q203	(Moraski-2016)
QcrB	M342T	Q-Y-A[2]	(Phumerian 2016)
QcrB	M342T	MOT	(Cleghorn-2018)
QcrB	M342T	PABs	(Chandrasekera-2015)
QcrB	M342V	AX-35	(Foo-2018)
QcrB	M342I	IPA	(Arora-2014)
QcrB	M342I	Q203	(Moraski-2016)
QcrB	M342I	TB47	(Waller 2023)
QcrB	M342I	AX-36	(Foo-2019)
QcrB	M342I	Q-Y-A[2]	(Phumerian 2016)
QcrB	A396T	IPA	(Arora-2014)
QcrB	A396T	Q203	(Moraski-2016)
QcrB	A296T	Q-Y-A[2]	(Phumerian 2016)
QcrA	L356W	JNJ-2901	This work
QcrA	L356W	Lanzoprazol	(Rybniker-2015)
QcrA	L356W	Q203	(Gries 2023)
QcrA	L356V	Quinazoline	(Lupien-2020)

Supplementary Table 1. Cytochrome bc resistance mutations. Reported mutations in Mtb cytochrome bc that give rise to resistance against various inhibitors. [1] P-P-D: Pyrrolo[3,4-c]pyridine-1,3(2H)-dione, [2] Q-Y-A: 2-(quinolin-4-yloxy)acetamide.

Name	Sequence (5'-3')	Role
p1	tgcctgcaggtcgactctagaggtggatcgcgctgttcgccacc	Amplification of *qcrCAB* operon
p2	gctcggtacccggggatctcagtgatgaccgttggtctccccgttgc	Amplification of *qcrCAB* operon
p3	ggtcaggagagcttcaacttcggtgagctTttcg	Delete Sap1 restricting site on qcrA
p4	cgaagttgaagctctcctgacccttgc	Delete Sap1 restricting site on qcrA
p5	atatatGCTCTTCtAGTgatcgTgctgttcgccaccatctacttcg	Subcloning *qcrABC* into pINIT
p6	tatataGCTCTTCaTGCgtgatgaccgttggtctccccgttgcc	Subcloning *qcrABC* into pINIT
p7	tctacaccgcgatgctgacg	Sequencing of *qcrCAB* operon
p8	gcctgatcaagaacccgtgg	Sequencing of *qcrCAB* operon
p9	tgtacagcttcatcatcctgc	Sequencing of *qcrCAB* operon
p10	gatgttcgagggctacttcggttactcg	Create $QcrB^{F156Y}$ mutation
p11	cgagtaaccgaagtagccctcgaacatcg	Create $QcrB^{F156Y}$ mutation
p12	atgggtatGcccgtcatcggcacctggCtgcactgggc	Create $QcrB^{I182M}$ and $QcrB^{M189L}$
p13	agtgcagccaggtgccgatgacgggcatacccatcg	Create $QcrB^{I182M}$ and $QcrB^{M189L}$
p14	atgatgtggaccgaGggtctggcccgtctgtggccg	Create $QcrB^{D309E}$ and $QcrB^{I112A}$
p15	ccacagacgggccagaccctcggtccacatcatgtagaagtccgg	Create $QcrB^{D309E}$ and $QcrB^{I112A}$
p16	accgaGggtctgGTccgtctgtgg	Create resistant mutation $QcrB^{A312V}$
p17	ccacagacggACcagaccCtcggtcc	Create resistant mutation $QcrB^{A312V}$
p18	gtcgcggtgggcGtgggcctggtgttcg	Create resistant mutation $QcrB^{M337V}$
p19	caggcccaCgcccaccgcgacccacacg	Create resistant mutation $QcrB^{M337V}$
p20	ctgctcgcacTGgggctgcccgtcctcg	Create resistant mutation $QcrA^{L349W}$
p21	gacgggcagcccCAgtgcgagcagaccttgg	Create resistant mutation $QcrA^{L349W}$
p22	gcgttcttcgcgatgatcacc	Verify $QcrB^{A312V}$ & $QcrB^{M337V}$
p23	gcctgatcaagaacccgtgg	Verify $QcrA^{L349W}$

Supplementary Table 2. Primers used in cloning of the cytochrome bc expression vector and generation of point mutants.