

Supplemental Information

Structural and mechanistic study of a novel inhibitor of *M. tuberculosis* cytochrome bc₁:aa₃

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	1	10	20	30	40
<i>M. smegmatis</i>	MDR.....IASMSQD.....SPDIKGTDAFGQTGVFGQPTDAELAEMSRE				
<i>M. tuberculosis</i>	MSRADDDAVGVPPTCGGRSDEEERRIVPGFNPQDGAKDGAKAATAVFPDPAALAAASNQ				

	50	60	70	80	90	100
<i>M. smegmatis</i>	ELVKLGKIDGVEITIFKEPRWPVPGTKAEKRTERTVAYWLMLGGLSGLALLLVFLFWPWE					
<i>M. tuberculosis</i>	ELLALGGKLDGVRITAYKEPRWPVPGTKAEKRAERSVAVWLMLGGVFGGLALLLVFLFWPWE					

	110	120	130	140	150	160
<i>M. smegmatis</i>	YQPFGSSEGEFLYSLATPLYGLTFGLSILSTIGTAVLFQKRFIPEEISVQDRHDGRSPSEVH					
<i>M. tuberculosis</i>	EKAADGSEDFLYSLTTPLYGLTFGLSILSTIATGAVLYQKRFIPEEISIQERHDGASREID					

	170	180	190	200	210	220
<i>M. smegmatis</i>	RKTVAANLTDALEGSTLKRKVLIGLSLGLCTGAFCTGLVAFITGGLKKNPWKPVPTAEG					
<i>M. tuberculosis</i>	RKTVVANLTDALEGSTLRRKVLIGLSFGVCMGAFCTGLVAFAGGLIKNPWKPVPTAEG					

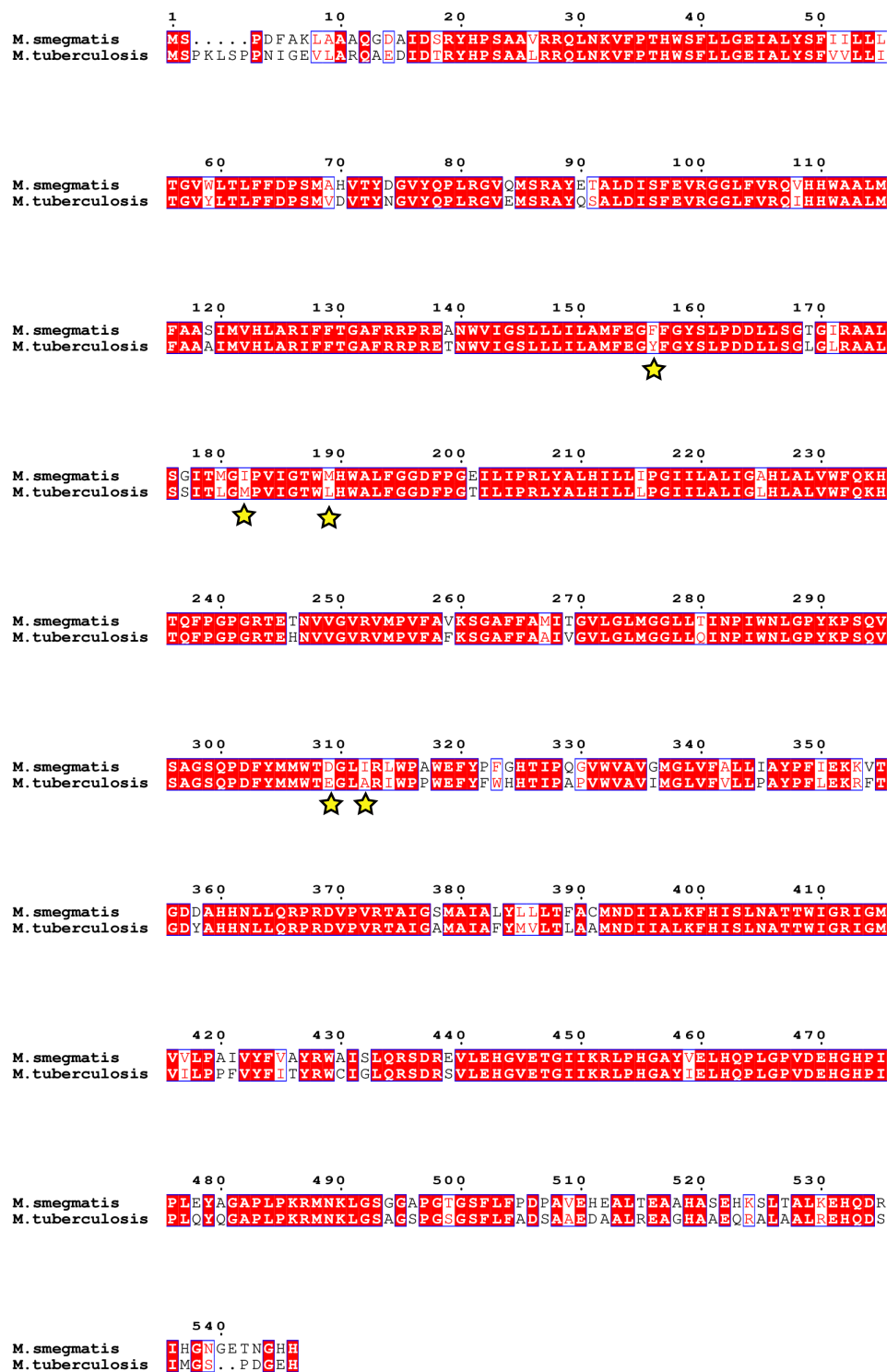
	230	240	250	260	270	280
<i>M. smegmatis</i>	KKAVLWTSGWTPRFKGETIYLARATGRPGESPFVKMRPEDMDAGGMETVFPWRESGDGDT					
<i>M. tuberculosis</i>	KKAVLWTSGWTPRVQGETIYLARATGTEDGPPFVKMRPEDMDAGGMETVFPWRESGDGDT					

	290	300	310	320	330	340
<i>M. smegmatis</i>	TVSEHKLTEIAMGVRRNPVMLIRIKPADMHRVIRKKGQESFNFGELFAYTKVCSHLGCPS					
<i>M. tuberculosis</i>	TVSEHKLQEIAMGIRNPVMLIRIKPSDLGRVVKRRKGQESFNFGELFAFTKVCSHLGCPS					

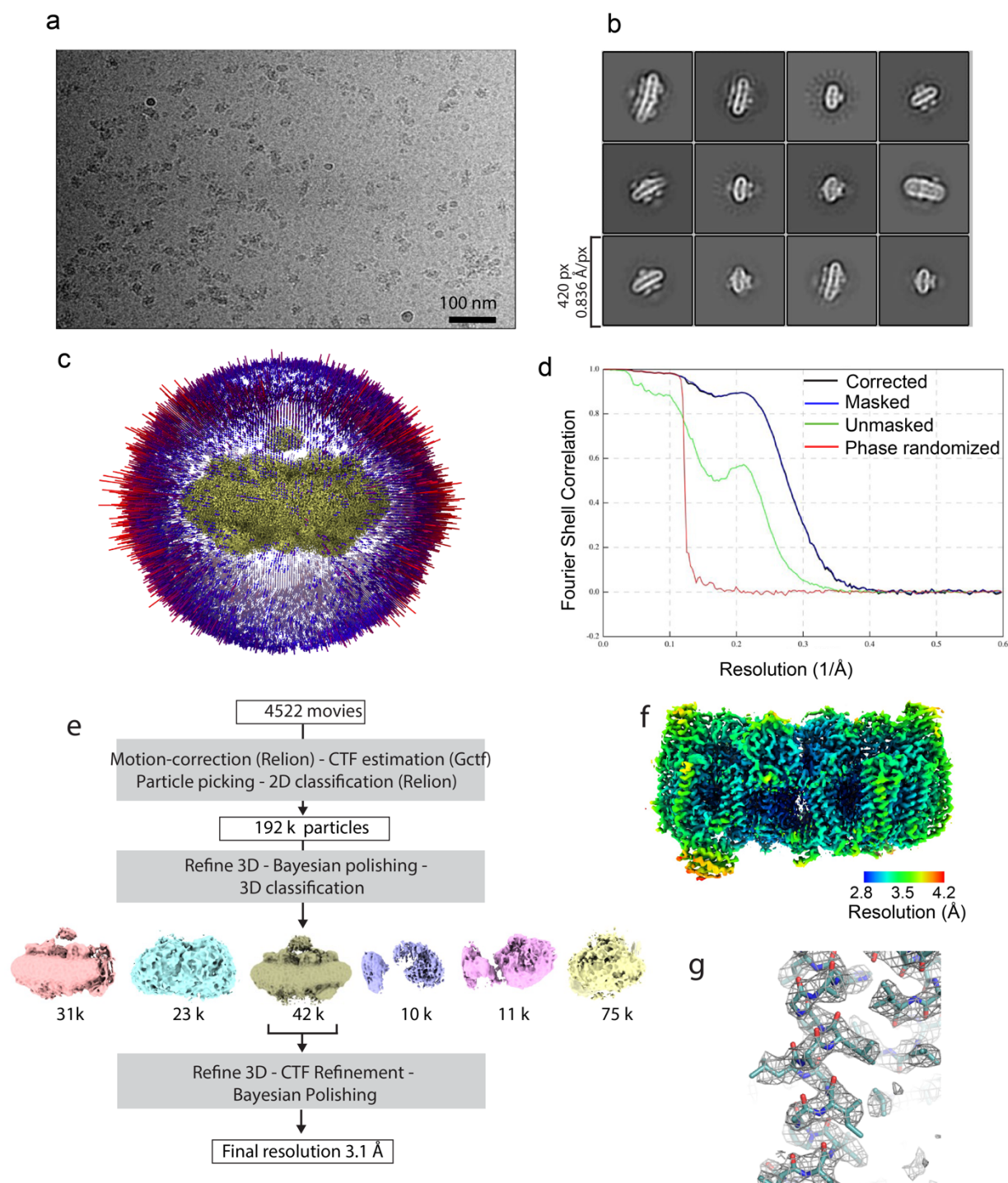
	350	360	370	380	390	400
<i>M. smegmatis</i>	SLYEQQTYRILCPCHQSQFDALFAKPIFGPAARALAQLPITIDE DGYLVANGDFVEPVG					
<i>M. tuberculosis</i>	SLYEQQSYRILCPCHQSQFDALFAKPIFGPAARALAQLPITIDTDGYLVANGDFVEPVG					

<i>M. smegmatis</i>	PAFWERKSS
<i>M. tuberculosis</i>	PAFWERTTT

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-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	tgcctgcaggctcactctagaggtggatcgcgctgttcgccacc	Amplification of <i>qcrCAB</i> operon
p2	gctcggtaacccggggatctcagtgatgaccgttggtctccccgttgc	Amplification of <i>qcrCAB</i> operon
p3	ggtcaggagagcttcaacttcggtgagctTttcg	Delete Sap1 restricting site on <i>qcrA</i>
p4	cgaagttgaagctctcctgacccttgc	Delete Sap1 restricting site on <i>qcrA</i>
p5	atatatGCTCTTCtAGTgatcgTgctgttcgccaccatctacttcg	Subcloning <i>qcrABC</i> into pINIT
p6	tatataGCTCTTCaTGCgtgatgaccgttggtctccccgttgc	Subcloning <i>qcrABC</i> into pINIT
p7	tctacaccgcgatgctgacg	Sequencing of <i>qcrCAB</i> operon
p8	gcctgatcaagaacccgtgg	Sequencing of <i>qcrCAB</i> operon
p9	tgtacagcttcacatcctgc	Sequencing of <i>qcrCAB</i> operon
p10	gatgttcgagggtacttcggttactcg	Create QcrB ^{F156Y} mutation
p11	cgagtaaccgaagtagccctcgaacatcg	Create QcrB ^{F156Y} mutation
p12	atgggtatGcccgtcatcgccacctggCtgactgggc	Create QcrB ^{I182M} and QcrB ^{M189L}
p13	agtgcagccaggtgccgatgacgggcataccatcg	Create QcrB ^{I182M} and QcrB ^{M189L}
p14	atgatgtggaccgaGggctctggcccgtctgtggccg	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	caggcccaCgcccaccgcgaccacacg	Create resistant mutation QcrB ^{M337V}
p20	ctgctcgcacTGgggctgcccgtcctcg	Create resistant mutation QcrA ^{L349W}
p21	gacgggcagcccCAgtgcgagcagaccttg	Create resistant mutation QcrA ^{L349W}
p22	gcgttcttcgcgatgacacc	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.