Mutations in *pepQ* Confer Low-level Resistance to Bedaquiline and Clofazimine in *Mycobacterium tuberculosis*

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Running Head: Resistance to Bedaquiline and clofazimine in *M. tuberculosis*

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Abstract

The novel ATP synthase inhibitor bedaquiline recently received accelerated approval for treatment of multidrug-resistant tuberculosis and is currently being studied as a component of novel treatment-shortening regimens for drug-susceptible and multidrug-resistant tuberculosis. In a limited number of bedaquiline-treated patients reported to date, upward shifts of ≥4-fold in bedaquiline MIC during treatment have been attributed to non-target-based mutations in *Rv0678* that putatively increase bedaquiline efflux through the MmpS5-MmpL5 pump. These mutations also confer low-level clofazimine resistance, presumably by similar mechanism. Here, we describe a new non-target-based determinant of low-level bedaquiline and clofazimine cross-resistance in *Mycobacterium tuberculosis*: loss-of-function mutations in *pepQ* (*Rv2535c*), a putative Xaa-Pro aminopeptidase. *pepQ* mutants were selected in mice by treatment with clinically relevant doses of bedaquiline, with or without clofazimine, and were shown to have bedaquiline and clofazimine MICs 4 times higher than parental H37Rv strain. Co-incubation with efflux inhibitors verapamil/reserpine lowered bedaquiline MICs against both mutant and parent strains to a level below the MIC against H37Rv in the absence of efflux pump inhibitors. However, qPCR revealed no significant differences in expression of *Rv0678*, *mmpS5* or *mmpL5* between mutant and parent strains. Complementation of a *pepQ* mutant with wild-type gene restored susceptibility, indicating loss of *pepQ* function is sufficient for reduced susceptibility both *in vitro* and in mice. Although the mechanism by which mutations in *pepQ* confer bedaquiline and clofazimine cross-resistance remains unclear, these results may have clinical implications and warrant further evaluation of clinical isolates with reduced susceptibility to either drug for mutations in this gene.

42 **Introduction**

43 Multidrug-resistant tuberculosis (MDR-TB) is a major threat to global control of
44 tuberculosis (TB). When multidrug resistance is not diagnosed, patients respond poorly to
45 standardized first-line regimens and additional resistance may develop. When MDR-TB is
46 diagnosed, current second-line regimens require prolonged treatment durations and are less
47 effective, more toxic and far more expensive than first-line therapy (1).

48 The diarylquinoline drug bedaquiline (B) received accelerated approval from the U.S.
49 Food and Drug Administration as part of combination therapy for MDR-TB when other
50 alternatives are not available (2). It is now being studied as a component of novel short-course
51 regimens for MDR as well as drug-susceptible TB (ClinicalTrials.gov Identifiers:
52 NCT02333799, NCT02193776, NCT02589782, NCT02409290, NCT02454205
53 [https://clinicaltrials.gov/]). For new drugs such as bedaquiline, it is essential to define and
54 catalog the mechanisms conferring bacterial resistance in order to design appropriate diagnostic
55 tests (including rapid molecular tests), to better manage the treatment of patients who fail therapy
56 or relapse after receiving the drug, and to conduct population level surveillance for changes in
57 drug susceptibility.

58 The principal mechanism of action of bedaquiline is inhibition of the mycobacterial ATP
59 synthase (3, 4). Strains selected *in vitro* for resistance to bedaquiline often have mutations in
60 *atpE*, which encodes the ATP synthase subunit to which bedaquiline binds (5). These target-
61 based mutations cause relatively large (i.e., 10-128x) shifts in MIC. However, it was noted that
62 many bedaquiline -resistant isolates selected *in vitro*, typically with smaller shifts in MIC, do not
63 have mutations in the ATP synthase complex (5). A new non-target-based mechanism conferring
64 low-level bedaquiline resistance and cross-resistance to clofazimine (C) was recently identified

among isolates from patients with delayed sputum culture conversion while receiving bedaquiline for MDR-TB, among mice treated with bedaquiline-containing combinations, and *in vitro* (6, 7). Although both bedaquiline and clofazimine (7) can select for non-target-based mutants *in vitro*, such mutants have not yet been isolated from mice or patients treated with clofazimine. The responsible mutations were found in *Rv0678*, which encodes a negative regulator of *mmpL5* and *mmpS5*. Increased transcription of these genes, which comprise a membrane transporter in the resistance-nodulation-division (RND) family, is putatively associated with increased efflux of bedaquiline and clofazimine.

Identification of mutations conferring cross-resistance to clofazimine and bedaquiline is important because clofazimine is a component of some short-course regimens currently under study for treatment of MDR-TB (8-10) and both drugs are being used together in the ongoing STREAM trial (ClinicalTrials.gov identifier: NCT02409290 [https://clinicaltrials.gov/]). Herein, we report a new genetic determinant of low-level bedaquiline and clofazimine cross-resistance in *M. tuberculosis*, conferred by mutations in *pepQ*.

Materials and Methods

Bacterial strains. *M. tuberculosis* H37Rv was passaged in mice, sub-cultured in Middlebrook 7H9 (Fisher Scientific) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) complex (Becton-Dickinson) and 0.05% Tween 80 (Sigma-Aldrich) and used for aerosol infection when the optical density at 600 nm ($O.D._{600}$) was approximately 1.0.

Antimicrobials. Pretomanid (Pa), moxifloxacin (M), bedaquiline and linezolid (L) were provided by the Global Alliance for Tuberculosis Drug Development (New York, NY), Bayer (Leverkusen, Germany), Janssen (Beerse, Belgium) and Pfizer (Groton, CT), respectively. Rifampin (R), isoniazid (H), pyrazinamide (Z), ethambutol (E), and clofazimine were purchased from Fisher or Sigma. Dosing formulations were prepared and maintained as previously described (11). All drugs were administered once daily by gavage, 5 days per week.

Aerosol infection. Female BALB/c mice (Charles River, Wilmington, MA) aged 4-to-6 weeks were infected by the aerosol route using the Inhalation Exposure System (Glas-col Inc., Terre Haute, IN). Mice were randomized to treatment groups (five mice per group per time point) after aerosol infection and were routinely sacrificed (i) on the day after infection to determine the number of colony-forming units (CFU) implanted in the lungs, (ii) on the day of treatment initiation to determine the pre-treatment CFU count, and (iii) at selected time points during and after treatment. Quantitative cultures of lung homogenates were performed in parallel on selective 7H11 agar with and without 0.4% activated charcoal to reduce drug carryover effects, as previously described (11). All procedures involving animals were approved by the Animal Care and Use Committee of Johns Hopkins University.

Efficacy of combinations containing bedaquiline and clofazimine in murine models of TB. Beginning 14 days after high-dose aerosol infection, as previously described (12), BALB/c mice received no treatment (negative controls) or treatment with the first-line regimen of RHZ (positive controls) or one of the following test regimens: bedaquiline (25 mg/kg) alone, the two-drug combination of bedaquiline plus clofazimine (20 mg/kg), or three-drug combinations of

bedaquiline plus clofazimine plus one of the following: rifampin (10 mg/kg), isoniazid, (10 mg/kg), pyrazinamide (150 mg/kg), ethambutol (100 mg/kg), moxifloxacin (100 mg/kg), pretomanid (50 mg/kg) and linezolid (100 mg/kg). Lung CFU counts were determined for all treatment groups after 4 weeks of treatment and, for mice receiving bedaquiline alone and bedaquiline plus clofazimine, also after 6 and 8 weeks of treatment.

MIC determination. Determination of bedaquiline and clofazimine MICs and the proportion of drug-resistant mutants was performed using the agar proportion method. Serial dilutions of lung homogenates, colony suspensions or a broth culture were inoculated in 500 µl aliquots onto 7H11 plates with and without the indicated drug. MICs were determined using doubling bedaquiline and clofazimine concentrations ranging from 0.003 to 0.25 µg/ml and 0.06 to 2 µg/ml, respectively. MIC was defined as the lowest drug concentration inhibiting at least 99% of the growth observed on drug-free control plates. To investigate the effect of efflux pump inhibitors on the susceptibility of the strains, the MIC of bedaquiline was also determined using the broth macrodilution method. Doubling concentrations of bedaquiline from 0.007 to 1 µg/ml were tested in the presence or absence of 40 µg/ml verapamil or 3 µg/ml reserpine. Briefly, tubes containing in 2.5 ml of 7H9 broth plus OADC with the above concentrations of bedaquiline were inoculated with 10^5 CFU of log phase culture of H37Rv or the B5 mutant. MIC was defined as the lowest concentration that prevented visible growth after 14 days of incubation at 37ºC. Controls with and without efflux inhibitors and bedaquiline were included for each test. The experiment was performed twice.

DNA sequencing. Genomic DNA from the parental wild-type strain and resistant mutants was extracted by using the cetyltrimethylammonium bromide (CTAB) protocol (13) and sonicated (Covaris, Inc.). The DNA library was constructed by using a genomic DNA sample preparation kit (Illumina, Inc.). The samples were sequenced on an Illumina Genome Analyzer II, which was operated in paired-end mode, collecting pairs of 51bp reads from opposite ends of ~250-350bp fragments. Image analysis and base-calling were done by using the Illumina GA Pipeline software (v0.3). Genome assembly was performed by a comparative assembly method using software developed in house. Briefly, reads were aligned to the genome of H37Rv as a reference sequence, and then local contig-building was used to identify insertions/deletions (14). Mutations in *pepQ* (Rv2535c) were confirmed by PCR amplification using specific primers (Table S1 in the supplemental material).

***In vivo* confirmation of low-level cross-resistance to bedaquiline and clofazimine.** BALB/c mice were infected with either the H37Rv parent strain or an isogenic strain (B5) with a non-synonymous *pepQ* mutation and randomized to receive no treatment, isoniazid (10 mg/kg) alone, bedaquiline (12.5, 25, or 50 mg/kg) alone, clofazimine (20 mg/kg) alone, or bedaquiline at 25 mg/kg plus clofazimine. Treatment began 4 days after infection and was administered for 4 weeks for all groups, except the bedaquiline 25 mg/kg groups, which received 8 weeks of treatment.

Complementation studies to confirm that a *pepQ* mutation is sufficient for bedaquiline and clofazimine cross-resistance. The B5 strain was complemented with pDT-Rv2535c (Figure S1 in the supplemental material) containing the wild-type *pepQ* gene from H37Rv.

Complementation was confirmed by showing loss of susceptibility to hygromycin and PCR for presence of the hygromycin resistance gene. Bedaquiline and clofazimine MICs were determined *in vitro*. Following aerosol infection with the H37Rv parent strain, the B5 mutant, or the B5: *pepQ* complemented strain, BALB/c mice were randomized to receive no treatment, isoniazid 10 mg/kg, bedaquiline 25 mg/kg, or clofazimine 20 mg/kg. Treatment began three days after infection and continued for 4 weeks before mice were sacrificed to determine lung CFU counts.

Expression analysis of *mmpS5*, *mmpL5* and *Rv0678*:

The H37Rv parent strain and the B5 mutant were grown to mid-log phase (OD_{600} = 0.6) in 7H9 broth and cells were pelleted by centrifugation. RNA was extracted by bead beating in Trizol and column purified according to the manufacturer's instructions (Qiagen, Valencia, California). RNA was reverse-transcribed using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). Real time PCR was performed with iQ SYBR Green supermix on a iCycler (BioRad, Hercules, CA) using primers specific for *Rv0676c*, *Rv0677c* and *Rv0678* and 16S rRNA (Table S1 in the supplemental material). All experiments were performed in biological and technical triplicates. Cycle threshold values were normalized against 16S rRNA expression, and fold-change was calculated by the $-2\Delta\Delta Ct$ method (15).

Expression, purification and activity assays of *pepQ*. The entire coding region of *Rv2535c* was cloned into pET28b expression vector (Novagen) containing an in-frame N-terminal 6x-His tag with the TEV cleavage site using the NdeI and HindIII restriction sites. The plasmid was transformed into BL21(DE3) *E.coli* cells for expression of *Rv2535c*. Cells containing the plasmid were grown at 37°C for 7 hours in LB medium with 50 µg/ml kanamycin followed by induction with 1 mM IPTG and grown overnight at 18°C. The cells were lysed via a

Microfluidizer M-100P (Microfluidics, Worcestershire, UK) in lysis buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 5% glycerol, DNase, and 2 mM $MgCl_2$) and centrifuged at 15,000 rpm for 1 hour. The supernatant was chromatographed over a His-tag affinity column (GE Healthcare) charged with Ni. The protein was eluted with 0 to 300 mM imidazole gradient and the 6x-His tag was cleaved using the tobacco etch virus protease. The final purification step was gel-filtration chromatography, and on the standardized s200 Superdex (GE Healthcare) column. *pepQ* eluted in two peaks: a large molecular weight aggregate that came out near the void volume, followed by a distinct peak with calculated molecular weight corresponding to a tetramer (~160 KDa). Formation of this tetramer is consistent with other related proteins, e.g. PDB ID 3Q6D. The protein was >95% pure, as observed by SDS-PAGE, and was concentrated to 7.5 mg/ml, flash frozen, and stored in dialysis buffer (50 mM Tris, pH 7, 50 mM NaCl, 5% glycerol, 1 mM DTT) at -80°C. The purified recombinant *pepQ* tetramer in 50mM Tris pH 7 was tested for both creatinase activity using the creatinase assay kit (Sigma) and for endopeptidase activity using commercially available fluorescent peptides. As the peptides were all labeled with a p-nitroanilide group the assay monitored for a shift in absorbance at 410 nm, resulting from the cleaved product.

Statistical analysis. CFU counts were $\log_{10}$ transformed before analysis. Group means for experimental treatment groups were compared with that of the standard treatment control by one-way analysis of variance (ANOVA) with Dunnett's post-test to adjust for multiple comparisons. All analyses were performed with GraphPad Prism v.4.01 (GraphPad, San Diego, CA).

Results

Antimicrobial Agents and Chemotherapy

Bedaquiline and clofazimine have additive activity against *M. tuberculosis* in mice but select for cross-resistant mutants. Using a well-established high-dose aerosol model of TB in mice, we evaluated the activity of novel drug combinations containing bedaquiline and clofazimine (Figure 1). Consistent with prior observations (11, 16, 17), treatment with the standard first-line RHZ regimen reduced the mean lung CFU count by 2.46 $\log_{10}$ over the first 4 weeks and treatment with bedaquiline alone reduced the lung CFU counts by an additional 1.08 $\log_{10}$. The addition of pyrazinamide and clofazimine each significantly increased the activity of bedaquiline ($p< 0.001$), whereas the addition of pretomanid had antagonistic effects ($p< 0.05$). The addition of rifampin, isoniazid, ethambutol or moxifloxacin did not significantly affect the activity of the bedaquiline-clofazimine combination, whereas addition of pyrazinamide significantly increased the activity ($p< 0.001$) and addition of linezolid or pretomanid reduced the activity ($p< 0.05$). After 8 weeks of treatment, 2 of 5 mice receiving bedaquiline alone had more than 3 log CFU in the lungs, while the mean log CFU count was 0.98 ± 0.53 in the remaining 3 mice. Likewise, 3 of 5 mice receiving bedaquiline plus clofazimine had the mean log CFU count of 2.12 ± 0.69, while the remaining 2 mice were culture-negative, as were all 5 mice treated for just 6 weeks (Figure 1 inset). Unlike those from mice with lower CFU counts at week 8, the isolates from the lungs of mice with higher CFU counts grew equally well on 7H11 agar with and without activated charcoal, indicating that their growth was not impaired by bedaquiline or clofazimine carried over in the lung homogenates.

Whereas bedaquiline and clofazimine MICs for the wild-type H37Rv parent strain were 0.03 and 0.25 µg/ml respectively, MICs for the isolates from the 2 mice treated with bedaquiline alone for 8 weeks (named B4 and B5) and the 3 mice treated with bedaquiline plus clofazimine for 8 weeks (named BC2, BC3 and BC4) with high, outlying lung CFU counts were 0.12-0.25

and 0.5-1 μg/ml, respectively, indicating that treatment with bedaquiline, with or without clofazimine, selected for strains with reduced susceptibility to both drugs.

Isolates with bedaquiline and clofazimine cross-resistance harbored mutations in *pepQ*. Duplicate samples of genomic DNA from four cross-resistant strains isolated in the first mouse experiment (i.e., B5, BC2, BC3 and BC4) were sequenced on an Illumina GenomeAnalyzer IIx in paired-end mode using a read length of 51 bp. The mean depth of coverage ranged from 118 to 161x (number of reads covering each site, averaged over the whole genome), and the completion was >99% (fraction of sites in 4.4 Mb genome covered by at least 1 read; sites lacking coverage were primarily restricted to PPE and PE_PGRS genes, due to very high GC content). The parental strain used in these experiments (H37RvJH) was also sequenced, to identify any differences from the public reference genome sequence for H37Rv (18). When compared to the sequence of the parental strain, only one mutation was observed in each strain (Table 1). All 4 strains had a mutation in *pepQ* (Rv2535c), which encodes a putative cytoplasmic peptidase. The mutations observed included 2 frameshift mutations (-C in Arg271, +C in Ala14) in mice treated with the bedaquiline-clofazimine combination, as well as a non-synonymous single nucleotide polymorphism causing a Leu44Pro mutation, obtained from a mouse treated with bedaquiline alone. All mutations were confirmed by PCR amplification and sequencing.

A *pepQ* mutant is virulent and less susceptible to bedaquiline and clofazimine in a murine TB model. Because transposon mutagenesis experiments have suggested that inactivation of *pepQ* may reduce the fitness of *M. tuberculosis* (19), and because the *pepQ* mutants displayed only low-level resistance to bedaquiline and clofazimine *in vitro*, we sought to confirm their

247 virulence and reduced susceptibility *in vivo*. We compared the parental H37Rv strain and the B5
248 mutant on the basis of their ability to multiply in untreated BALB/c mice and their susceptibility
249 to treatment with isoniazid (positive control), bedaquiline, and/or clofazimine. The aerosol
250 infectious dose was approximately 1 $\log_{10}$ higher for the parent strain compared to the B5 mutant
251 (Figure 2). By 4 weeks post-infection, untreated (UT) control mice infected with H37Rv had
252 died and therefore none were available for CFU counts (although lung CFU counts are typically
253 > 8 $\log_{10}$, or approximately 4 $\log_{10}$ higher than baseline, at the time of death). Untreated mice
254 infected with B5 did not die within 4 weeks of infection because the infectious dose was not high
255 enough. However, the approximately 4 $\log_{10}$ increase in lung CFU counts over this time period is
256 evidence that the strain multiplies more-or-less normally in mice. The treatment with isoniazid
257 (positive control) was at least as bactericidal against the B5 strain as against H37Rv, reducing the
258 lung CFU counts by more than 1 $\log_{10}$ in both strains. Dose-dependent activity of bedaquiline
259 was observed against both strains. However, whereas bedaquiline 12.5 mg/kg was bacteriostatic
260 and 50 mg/kg reduced the lung CFU counts by more than 2 $\log_{10}$ against H37Rv, even 50 mg/kg
261 of bedaquiline did not fully inhibit multiplication of the B5 mutant but rather allowed a nearly 2
262 $\log_{10}$ increase in CFU counts. Clofazimine enabled growth of both strains in this acute infection
263 model, as previously observed (20), but was more effective against the H37Rv strain compared
264 to the B5 mutant.
265
266 **Complementation of *pepQ* restores susceptibility to bedaquiline and clofazimine.**
267 Complementation of the B5 and BC2 strains with the wild-type *pepQ* gene from H37Rv restored
268 the susceptibility of the strains to bedaquiline, as confirmed by the MIC of 0.03 µg/ml, which
269 was no different from the parental H_{37}Rv control. Following this, 3 groups of mice were infected

with $H_{37}Rv$, the B5 strain, or the B5::*pepQ* strain and initiated on treatment with isoniazid 10 mg/kg, bedaquiline 25 mg/kg, or clofazimine 20 mg/kg four days later. In untreated mice, lung CFU counts increased by approximately 4 $\log_{10}$ over the subsequent 4 weeks (Figure 3) and were accompanied by visible lung lesions (Figure S2 in the supplemental material). Isoniazid was bactericidal, with the greatest effect against the B5 strain, and prevented the formation of lung lesions. Like isoniazid, bedaquiline reduced the mean lung CFU counts by 0.5-1 $\log_{10}$ CFU against H37Rv and B5:*pepQ*, and it did prevent formation of macroscopic lung lesions in mice infected with the B5 mutant (Figure S2 in the supplemental material). However, bedaquiline allowed a >1 $\log_{10}$ increase in mean CFU counts of the B5 mutant. Clofazimine, which has a relatively poor activity in this acute infection model when given alone (20), allowed multiplication of all strains but significantly reduced the multiplication of the $H_{37}Rv$ ($p < 0.05$) and B5:*pepQ* ($p < 0.05$) strains, but not the B5 strain ($p > 0.05$) when compared to no treatment. In addition, clofazimine prevented formation of lung lesions in mice infected with H37Rv and the B5:*pepQ* complemented strain, but not the B5 strain (Figure S2 in the supplemental material). These results clearly demonstrate that complementation with the wild-type *pepQ* gene was sufficient to restore susceptibility to bedaquiline and clofazimine in the B5 mutant and thus confirm the loss of *pepQ* function is sufficient for low-level resistance to bedaquiline and clofazimine.

Structural analysis of *M. tuberculosis pepQ*. *pepQ* is a 372-amino-acid protein that has two domains: a ~100 aa N-terminal alpha/beta domain, and a ~250-aa C-terminal peptidase domain (Figure 4). Based on homology to Ypdf in *E. coli* (e.g. 37% amino acid identity over 363 residues), *M. tuberculosis pepQ* is predicted to be a proline-specific aminopeptidase (prolidase),

active on substrates with Xaa_1-Pro_2 at the amino terminus. The C-terminal domain of YpdF houses the catalytic activity, and is homologous to the *E. coli* methionine aminopeptidase (MetAP; 29% aa identity) (21). However, MetAP lacks the N-terminal domain. In enzyme assays, YpdF was found to have weak activity on substrates with an N-terminal methionine (Met-Xaa) if the second aa is alanine, proline or serine, and higher activity for other substrates with a proline in the second position (Xaa-Pro) (22). In *E. coli,* YpdF is encoded in an operon with YpdE, which is a methionine aminopeptidase. YpdF and YpdE are proposed to work in concert to degrade proteins, with YpdE removing amino acids from the N-terminus until a proline is encountered in the 2-position, and YpdF removing the block (22). *M. tuberculosis pepQ* also has homology (~30% aa identity) to other *E. coli* proline aminopeptidases, *pepP* and *pepQ*, which have both the catalytic C-terminal domain and a non-catalytic N-terminal domain that plays a role in oligomerization and substrate specificity. *pepP* and similar aminopeptidase P enzymes (EC 3.4.11.9) may preferentially hydrolyze the Xaa-Pro bond at the terminus of larger oliogopeptides (but also dipeptides) and can discriminate the 3rd and 4th residues in their catalytic specificity.

Proline aminopeptidases have similar 2-domain architecture to creatinases (which catalyze a similar reaction, hydrolysis of creatine into sarcosine and urea), although peptidase activity of the former is metal-ion dependent and they bind cations in the active site (21), whereas creatinase activity does not require metal ions (23, 24). The mutations selected by treatment with bedaquiline ± clofazimine in mice occurred in both domains. The frameshift mutation in Ala14 and the non-synonymous mutation L44P occur in the N-terminal domain, and the frameshift mutation in Arg271 occurs in the catalytic domain. Figure 4 illustrates where these mutations fall in the domain structure of *pepQ*. To better understand the role of these mutations,

316 we used Phyre2 (25) to build a homology model of the 3D atomic structure *pepQ*. Phyre2
317 detected the greatest homology to a proline aminopeptidase from *B. anthracis* (PDB: 3q6d, 39%
318 amino acid identity; deposited by Midwest Center for Structural Genomics). Phyre2 created a
319 hidden Markov model based on a Psi-BLAST alignment of closely related sequences and used it
320 to optimally thread the *M. tuberculosis pepQ* amino acid sequence onto the 3q6d structure as a
321 template, followed by loop optimization and energy minimization (25). The active site can be
322 inferred to be in a pocket in the C-terminal domain containing a Ca^{2+} metal ion, in the same
323 position as the dinuclear divalent cation sites in *pepP* (1a16) and MetAP (3mat), which is
324 involved in catalyzing the cleavage of the scissile peptide bond. The cations are coordinated by
325 D221, D232, E339, E325, and His296 (Mtb *pepQ*), all perfectly conserved in all of these
326 structures. Based on the homology model, Leu44 of *M. tuberculosis pepQ* appears buried in the
327 core of the N-terminal domain (on a beta strand that shows a high level of conservation with *E.*
328 *coli pepP* and both prokaryotic and eukaryotic prolidases, (26) not in close proximity to the
329 active site. Thus, L44P is likely a structure-destabilizing mutation, as is the frameshift mutation
330 in Ala14. A frameshift in Arg271 would truncate half the C-terminal domain (splitting the
331 pseudo-symmetric alpha/beta "pita-bread" fold in half and destroying the active site).

332 The *pepQ* model can be superimposed with the crystal structure of the *E. coli* MetAP
333 protein (3mat), which was complexed with a peptide inhibitor (bestatin analog). The mechanism
334 and specificity determinants of *E. coli* MetAP have been well characterized (21). MetAP has a
335 dinuclear cation site (2 Co2+) in the same location as in 3q6d. The N-terminal residue of the
336 peptide inhibitor of MetAP binds in a P1 pocket, and the second residue binds in a P2 pocket,
337 with the polar backbone atoms of the scissile bond between them coordinating the cations. The
338 residues of *M. tuberculosis pepQ* lining the P1 part of the pocket are considerably different than

MetAP. For example F190 is replaced with Cys in MetAP, and I302 is replaced with F. This could potentially explain differences in specificity for the N-terminal residue between prolidases and methionine aminopeptidases (27). While the inhibitor in the 3mat structure mimics Met_1-Ala_2, YpdF would be expected to bind Xaa_1-Pro_2 as a prolidase. The putative position of the P2 pocket can also be inferred by superimposing the crystal structure of *E. coli pepP* in complex with the inhibitor Pro-Leu (PDB: 1a16), which putatively mimics the 2nd and 3rd residues of a peptide substrate. In both MetAP and *pepP*, the P2 pocket is adjacent to residue His-204. While the homology between *M. tuberculosis pepQ* and *E. coli pepP* is relatively low (31% aa identity), nearly every residue in the vicinity of the active site was either identical or highly conserved. The residues lining the P1 pocket are F190 (Y), I302 (V), H303, T341 (D), T232, and V398 (L), with residues of *E. coli pepP* shown in parentheses. The residues lining P2 are T234, I193, R337, H204, P203 (L), and H292 (Figure 5). Thus we expect *M. tuberculosis pepQ* to be able to coordinate metal ions similar to *E. coli pepP*, and the specificity of *M. tuberculosis pepQ* to be very similar to *E. coli pepP*, i.e. a proline aminopeptidase (Xaa-Pro).

Enzymatic characterization of *M. tuberculosis pepQ*.

Recombinant purified *M. tuberculosis pepQ* exhibited no creatinase activity (data not shown). Of the 6 proline-containing peptide substrates tested, only Arg-Pro-p-nitroanilide showed modest cleavage activity. Several substrates with an N-terminal methionine were also tested. However, none of these showed cleavage, suggesting *pepQ* is not a methionine aminopeptidase.

Mass spectrometry showed that recombinant *M. tuberculosis pepQ* does not bind to or modify the structure of bedaquiline or clofazimine. Over-expression of *M. tuberculosis pepQ* in *Mycobacterium smegmatis* did not significantly alter the MIC of bedaquiline or clofazimine.

This is not surprising as the mutations presumably result in loss of function. Taken together, these results suggest *pepQ* is not a target or activating enzyme for either drug.

Resistance mediated by *pepQ* mutation may be associated with increased drug efflux, but this is not due to up-regulation of *mmpL5* and *mmpS5* expression.

Addition of verapamil and reserpine reduced the MIC against the H_{37}Rv parent strain from 0.03 to 0.007 µg/ml and 0.015- 0.03 µg/ml respectively. Both efflux inhibitors reduced the MIC of both the B5 mutant and the H37Rv parent to the same level that was below the MIC against the parent strain in the absence of the efflux inhibitor, indicating a possible role of one or more efflux pumps in the mechanism of low-level bedaquiline resistance mediated by loss-of-function mutations in *pepQ* (Table S2 in the supplemental material). Further, qPCR for the genes involved in the efflux-mediated resistance reported in previous studies did not show any significant fold change in transcription levels (Table S3 in the supplemental material).

Discussion

Bedaquiline is a promising new drug for the treatment of TB. Its limited clinical usage to date has provided little opportunity to select for drug-resistant mutants or to assess the impact of such mutants on treatment outcomes. *In vitro*-selected target-based mutations in *atpE* confer high-level resistance to bedaquiline (e.g., 16-128x increase in MIC)(5, 6). However, to our knowledge, no *atpE* mutant has been isolated from a patient treated with bedaquiline. On the other hand, non-target mutations in *Rv0678* conferring low-level resistance to bedaquiline and clofazimine were recently described in sputum isolates with at least 4-fold increases in bedaquiline MIC after treatment including bedaquiline (6). More recently, *Rv0678* mutants were

identified in the sputum of both MDR-TB and drug-susceptible TB patients that had not received bedaquiline or clofazimine (28). Although these *Rv0678* mutations cause relatively small (2-8x) increases in bedaquiline and clofazimine MICs compared to mutations in *atpE*, their selection during combination therapy in TB patients and in mice is a cause of concern (6). In mice, *Rv0678* resistant mutants may be selected with 8 weeks of bedaquiline monotherapy (D. Almeida, unpublished observation) but emerge only late in the course of combination therapy and are eventually cleared, presumably by the action of companion agents (6). Clinically, these mutants emerged almost entirely among patients with pre-XDR- and XDR-TB (6) but their isolation has not clearly been associated with poorer clinical outcomes among patients receiving bedaquiline with more effective companion agents (29). These results underscore the importance of appropriate combination therapy and adherence, and the risk of premature discontinuation of therapy.

The present study provides the first evidence of non-target mutations in *pepQ* conferring low-level resistance to bedaquiline and cross-resistance to clofazimine. Zhang et al (30) recently reported *in vitro* selection of a *pepQ* mutant in *M. tuberculosis* with clofazimine. However, they did not report the results of direct susceptibility testing with clofazimine or bedaquiline, or confirm the causative role of *pepQ* mutation in clofazimine resistance. Like mutations in *Rv0678* (6), the observed *pepQ* mutations produce modest increases (up to 4-fold) in bedaquiline and clofazimine MICs and reduce the efficacy of bedaquiline and clofazimine *in vivo*. However, these mutations did not result in complete resistance to these drugs, as increasing doses of bedaquiline and, to a lesser extent, combining bedaquiline and clofazimine were associated with greater anti-tuberculosis effect. These data and the additive bactericidal and sterilizing effects of bedaquiline and clofazimine against drug-susceptible bacilli (31) indicate that, despite the

presence of two shared resistance mechanisms, the combined use of clofazimine and bedaquiline may be advantageous. However, these two drugs will not protect each other against the emergence of resistance and it is therefore important to use them in combination only if they are protected from emergence of resistance by additional antibiotics that are not substrates of the same efflux pumps. Both *Rv0678* and *pepQ* mutants are selected and able to grow in mice treated with bedaquiline, despite plasma bedaquiline concentrations exceeding the MIC against these resistant (11, 32) organisms. This may be due to the high protein binding of bedaquiline, which results in free drug concentrations below the MIC at the site of infection. *Rv0678* mutants and *pepQ* mutants may be selected preferentially over *atpE* mutants *in vivo* during bedaquiline treatment because they strike the right balance between reduced susceptibility to bedaquiline and maintenance of fitness.

Our findings may have implications for breakpoint selection for bedaquiline susceptibility testing. Although the bedaquiline MIC against the *pepQ* mutants is below the provisional susceptibility breakpoint of 0.5 µg/mL proposed by EUCAST (33), monotherapy with clinically relevant bedaquiline doses in mice infected with a *pepQ* mutant had limited efficacy. These findings and the limited clinical data available to date warrant careful monitoring of bedaquiline MICs and treatment outcomes and further consideration whether a lower breakpoint would better predict patient response to bedaquiline treatment. Polymorphisms in *Rv0678* have recently been observed among isolates from patients that have not received bedaquiline or clofazimine and are not always associated with bedaquiline MICs that exceed the provisional breakpoint (28), and it remains unclear whether such *Rv0678* mutations are associated with poorer clinical outcomes among patients receiving bedaquiline-containing regimens (6, 29). Thus, the clinical impact of the small shift in MIC conferred by *pepQ*

mutations remains to be determined. Further surveillance is required to identify *Rv0678* and *pepQ* mutants and to correlate their presence with MICs and clinical outcomes among patients receiving therapy that includes one or both drugs.

It is interesting to note that single nucleotide polymorphisms in *pepQ* were occasionally observed in clinical isolates that we have sequenced. In particular, *pepQ*:Ser66Pro was observed in two XDR-TB isolates (X16 and X23; Beijing strain family) and an MDR-TB isolate (R1792; LCC strain family) from the Western Cape of South Africa, all isolated prior to the introduction of bedaquiline (34). No other strains were observed to have mutations in *pepQ* among over 50 clinical isolates from South Africa and South America. It is possible that use of clofazimine to treat MDR-TB in this region could have contributed to selective amplification of these mutants. However, this seems unlikely given limited usage of clofazimine in South Africa during the period in which such selection would have occurred. The clinical isolates with polymorphisms in *pepQ* were not available for drug-susceptibility testing. However, the presence of *Rv0678* and *pepQ* mutations in isolates from patients naïve to bedaquiline and clofazimine raises the possibility that they may also be selected by other drugs used to treat TB or other infections.

What is the function of *pepQ* in *M. tuberculosis* and how might loss-of-function result in reduced susceptibility to bedaquiline and clofazimine? While further characterization of the enzymatic activity of *pepQ* is clearly required before drawing conclusions, the genomic and structural characterization described here suggests *pepQ* is a proline-specific aminopeptidase and ortholog of *pepP* in *E.coli* and *L. lactis* and PapA (YqhT) in *Bacillus subtilis* (35). This is supported by homology in both genetic sequence and predicted protein structure. Although the DNA sequence homology is not particularly high, there is a high degree of conservation of amino acids in the predicted active site, including strict conservation of metal-binding residues

between *pepQ* and *pepP* from both *E.coli* and *L. lactis*. Moreover, each of these orthologous genes is situated next to and co-expressed with elongation factor P (EF-P; Rv2534c), one of the few universally conserved elongation factors (35). Conserved homologs also exist in other pathogenic mycobacteria (*M. bovis*, *M. leprae*, *M. paratuberculosis* (36).

Proline-specific aminopeptidases are one of only 80 enzymes conserved in the 3 major kingdoms of life (37). Such high conservation may stem from the exceptional conformational rigidity that proline residues introduce into peptide chains, which presents challenges to proteolytic enzyme systems. For example, in *E. coli*, the essential methionine aminopeptidase (PepM) responsible for co-translational N-terminal methionine excision readily liberates the N-terminal methionine when proline or an amino acid with similarly small side chain occupies the penultimate position. However, a proline in the third position inhibits PepM activity (38, 39), making *pepP* required for efficient N-terminal methionine excision in such cases (40).

The conformational constraints of poly-proline motifs also present challenges during ribosomal peptide synthesis by stalling translation. As EF-P is required to prevent such stalling and promote efficient translation of poly-proline-containing peptides (41, 42), the genomic organization of a putative proline-specific aminopeptidase like *pepQ* with EF-P is likely no coincidence. Work in Gram-negative enteric pathogens has revealed that proteins with EF-P target motifs (and hence dependent on EF-P for optimal translation) are enriched for metabolic enzymes, membrane-associated proteins, transporters and two-component regulatory systems (43). The same appears true for *M. tuberculosis*. This system may have evolved for rapid remodeling of the cellular proteome in response to changing environmental conditions, such as carbon source (44, 45). The highly conserved organization of genes for proline-specific aminopeptidases and EF-P in bacteria thus supports a potential role for *pepQ* in regulating

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maturation and/or turnover of specific proteins in a manner that is sensitive to the metabolic status of *M. tuberculosis*.

Proline-specific aminopeptidases also have important roles in the utilization of exogenous and endogenous proteins as sources of essential amino acids useful for protein synthesis, energy production, osmoprotection and recycling of reduced cofactors. Proline itself is increasingly recognized as having a critical role in energy metabolism, redox control and bacterial virulence (46). In early transposon mutagenesis studies (19), disruptions of *pepQ* were observed to cause a growth defect (thus neither absolutely essential nor non-essential *in vitro*). More recent high-resolution transposon-mutagenesis studies suggest that *pepQ* is conditionally essential when grown on glycerol as a carbon source, but not essential when grown on cholesterol (p-value <0.01 for comparison of transposon insertion counts) (47). This is interesting given recent evidence linking mycobacterial proline catabolism to protection from the toxic effects of methylglyoxal produced during growth on glycerol (48). If mutation of *pepQ* reduces intracellular availability of proline, this might increase susceptibility to methylglyoxal and reduce growth on glycerol.

The mechanism by which *pepQ* mutations result in reduced susceptibility to bedaquiline and clofazimine is unclear. Rv0678 mutations appear to confer cross-resistance to bedaquiline and clofazimine via de-repression of *mmpL5-mmpS5* (*Rv0676c-Rv0677c*), which encode an RND transporter capable of exporting bedaquiline and clofazimine (6, 7). Reversion of bedaquiline susceptibility of a *pepQ* mutant to that of its wild-type parent in the presence of efflux pump inhibitors suggests drug efflux is involved in the mechanism of resistance. However, unlike the mutations in Rv0678, the mutation in *pepQ* is not associated with over-expression of *mmpL5* or *mmpS5*. One possibility is that *pepQ* mutations increase efflux through this transporter by a

different means, such as preventing degradation of MmpL5, which contains a Val-Pro-Pro stretch near the amino terminus.

In conclusion, we demonstrate that loss-of-function mutations in *pepQ* confer reduced susceptibility to bedaquiline and clofazimine. While the shift in susceptibility is relatively small, it is of similar magnitude to mutations in *Rv0678* that have been selected in patients and mice receiving combination therapy including bedaquiline. Therefore, these results should provide the impetus to include *pepQ* in the genetic analysis of bedaquiline- and clofazimine-resistant strains identified during clinical usage. Further study of the biological function of *pepQ* in *M. tuberculosis* and the mechanism by which *pepQ* mutations confer reduced susceptibility is clearly warranted.

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692 **Table 1.** Sequencing results for the resistant strains.

Mouse number	Treatment	Coverage	Completion	MIC (in µg/ml)		Mutation
				B	C	
B5	B	142.1x	99.25%	0.12	0.5-1	*pepQ*: L44P
BC2	B+C	118.4x	99.20%	0.12	0.5-1	*pepQ*: +c in Ala14
BC3	B+C	154.3x	99.43%	0.12	0.5-1	*pepQ*: +c in Ala14
BC4	B+C	161.1x	99.14%	0.12	0.5-1	*pepQ*: -c in Arg271

693 B = bedaquiline; C = clofazimine

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698 **Figure 1.** Mean lung $\log_{10}$ CFU counts (± SD) after 4 and 8 weeks of treatment with
699 bedaquiline- and clofazimine-containing regimens. Main figure does not include results from
700 mice in which bedaquiline and clofazimine cross-resistant mutants were selected after 8 weeks of
701 treatment: 2 and 3 mice from the B and BC groups with mean lung CFU counts of 3.22 ± 0.16
702 and 2.12 ± 0.69 $\log_{10}$, respectively (inset). The CFU counts at the time of treatment initiation are
703 indicated by Day 0. Abbreviations: R, rifampin; H, isoniazid; Z, pyrazinamide; B, bedaquiline;
704 C, clofazimine; L, linezolid; Pa, pretomanid; E, ethambutol; M, moxifloxacin.
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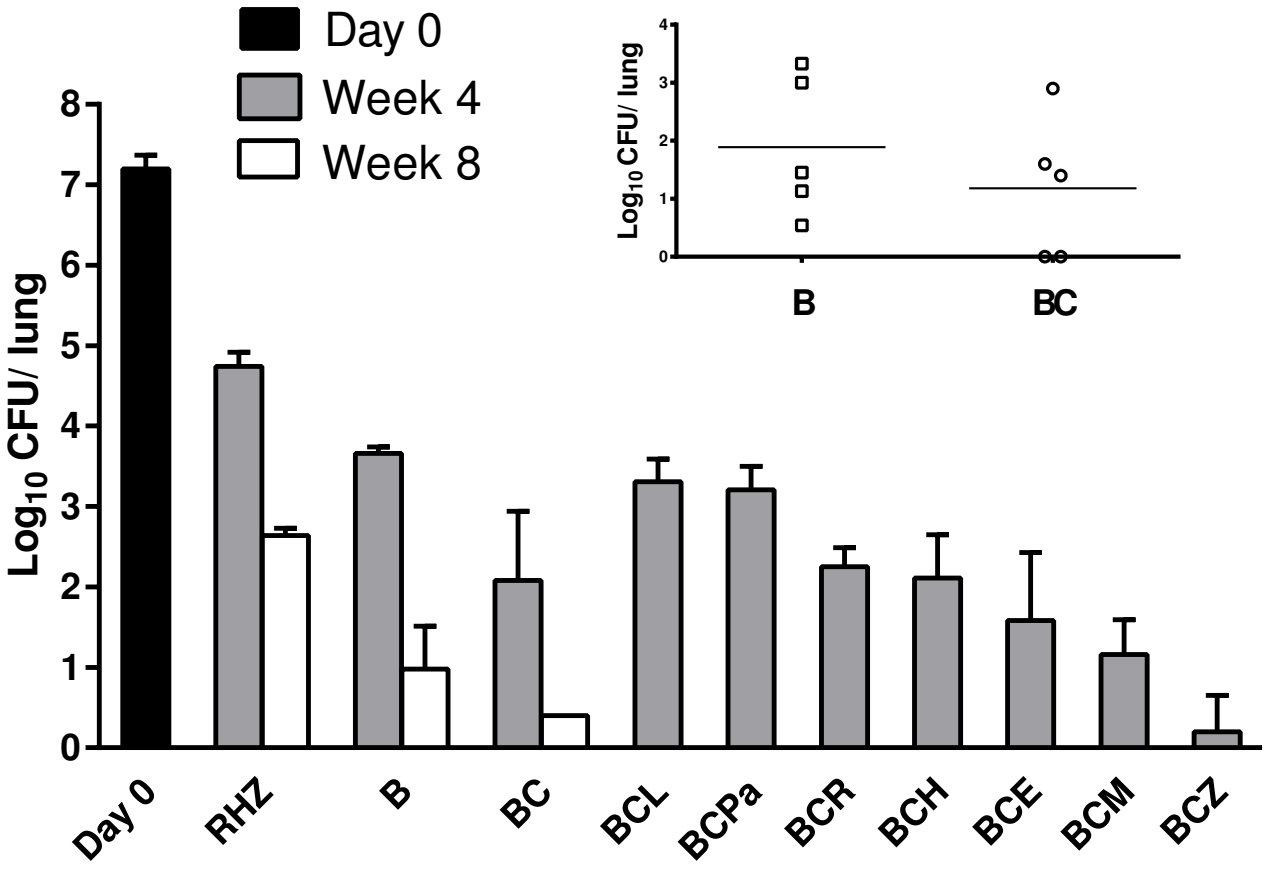
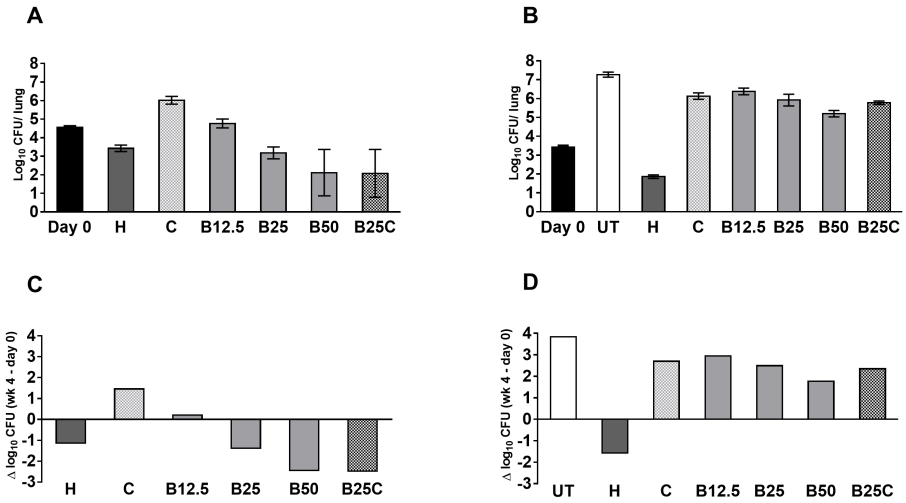
710 **Figure 2.** Mean lung $\log_{10}$ CFU counts (±SD) (upper panels) and change in CFU counts (Week 4
711 - Day 0) (lower panels) after 4 weeks of treatment in mice infected with the parental H37Rv
712 strain (left panels) or the B5 mutant (right panels). CFU counts were not determined for
713 untreated mice infected with the parental strain, which required euthanasia prior to the
714 predetermined endpoint. Abbreviations: UT, untreated; H, isoniazid (10 mg/kg); C, clofazimine
715 (20 mg/kg); B, bedaquiline (12.5, 25 or 50 mg/kg).
716
717 **Figure 3.** Mean lung $\log_{10}$ CFU counts (±SD) (upper panels) and change in lung CFU counts
718 (Week 4 – Day 0) (lower panels) after 4 weeks of treatment in mice infected with the parental

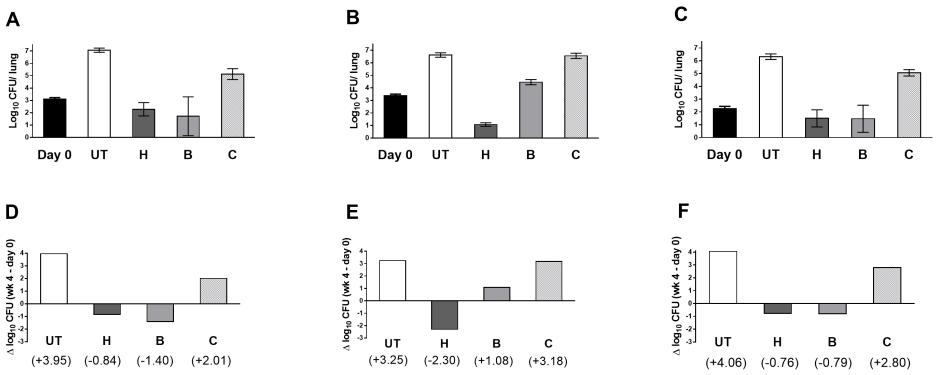
H37Rv strain (left panels), the B5 mutant (middle panels), or the complemented B5:*pepQ* strain (right panels). Abbreviations: UT, untreated; H, isoniazid (10 mg/kg); C, clofazimine (20 mg/kg); B, bedaquiline (25 mg/kg).

Figure 4. Location of mutations and domains in *pepQ*.

Figure 5. Multiple amino acid sequence alignment for *M. tuberculosis pepQ*, *L. lactis pepP*, and *E. coli pepP*, constructed using ClustalW. Residues coordinating the Mn^{2+} ions are shown in red. Asterisks indicates positions which have a single, fully conserved residue. Colons indicate conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. Periods indicate conservation between groups of weakly similar properties - scoring ≤ 0.5 in the Gonnet PAM 250 matrix.







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