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Two-Way Regulation of MmpL3 Expression Identifies and Validates Inhibitors of MmpL3 Function in *Mycobacterium tuberculosis*

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**ABSTRACT:** MmpL3, an essential mycolate transporter in the inner membrane of *Mycobacterium tuberculosis* (Mtb), has been identified as a target of multiple, chemically diverse antitubercular drugs. However, several of these molecules seem to have secondary targets and inhibit bacterial growth by more than one mechanism. Here, we describe a cell-based assay that utilizes two-way regulation of MmpL3 expression to readily identify MmpL3-specific inhibitors. We successfully used this assay to identify a novel guanidine-based MmpL3 inhibitor from a library of 220 compounds that inhibit growth of Mtb by largely unknown mechanisms. We furthermore identified inhibitors of cytochrome bc1 oxidase as one class of off-target hits in whole-cell screens for MmpL3 inhibitors and report a novel sulfanylacetamide as a potential QcrB inhibitor.

**KEYWORDS:** targeted whole-cell screen, mycolic acids, antibiotics, molecular genetics, drug discovery, respiration

*Mycobacterium tuberculosis* (Mtb), etiologic agent of tuberculosis (TB), is the leading cause of adult mortality due to a single infectious agent in both healthy and HIV-infected individuals. In 2019, the World Health Organization (WHO) estimated a total of 1.7 billion people to be infected with *Mtb* and at risk of developing the disease.1

The preferred treatment for drug-susceptible TB requires at least four drugs with toxic side effects, administered routinely for a minimum of two months during the intensive phase and at least two drugs during the continuation phase of four months.1 This leads to poor compliance, which accelerates the emergence of drug-resistance, and causes relapse of disease.2,3 Reducing the global TB epidemic necessitates development of new therapeutics to shorten the duration of treatment and to be effective against the drug-resistant forms of infection.

To facilitate TB drug discovery efforts, whole-cell screens (WCS) against *Mtb* have identified the highest number of novel pharmacophores with potent antimycobacterial activity.4,5 Interestingly, multiple chemical entities discovered using this approach primarily target MmpL3, an essential lipid transporter in mycobacteria.6–14 A member of the resistance nodulation and division (RND) superfamily of transporters, MmpL3 facilitates transport of trehalose monomycolate (TMM) into the periplasmic space where it is modified and assembled into the outer layer of the mycobacterial cell envelope.15–17 Chemical and genetic inhibition of MmpL3 leads to a sharp decline in mycolate transport, bacterial growth, and viability, both in vitro and in the mouse model of TB, rendering it a significant target of therapeutic interest.6–10,18–21 Anti-MmpL3 pharmacophores, including adamantyl urea,6 pyrroles,7 indolecarboxamides,8–10 diamines,6,22 tetrahydropyrazolopyrimidines (THPPs), and spirocycles (Spiros),8,23 are active against drug-resistant strains of Mtb. In addition, several of these scaffolds synergize with existing antitubercular drugs, for example, rifampicin, suggesting that inhibition of MmpL3 could shorten TB treatment.8,10,24–29 Among these pharmacophores, the diamine SQ109 has successfully completed phase 2b-3 of clinical trials30,31 and others such as indolecarboxamides, pyrroles, and THPPs exhibit favorable pharmacokinetic profiles and efficacy in murine models of infection.8,10

To date, identification and characterization of MmpL3 inhibitors relies on integration of cell-based screenings of chemical libraries with genetic techniques, such as isolating resistant mutants and performing cross-inhibition experiments, followed by chemical-radiolabeling to assess TMM levels to...
identify the target. While informative, most of these methods are laborious, compound-intensive, and difficult to implement in a high-throughput format, hence limiting their utility. The need to simplify identification of MmpL3 inhibitors was recently addressed by an elegant study, which utilized a pool of 24 unique mmpL3 mutants. Each mutant caused resistance against a subset of the known MmpL3 inhibitors. This increases screening efficiency significantly, but only for compounds susceptible to already known resistance mutations.\(^{32}\) Furthermore, a cohort of MmpL3 inhibitors, including THPPs, seems to impede bacterial growth either by inhibition of multiple targets\(^ {23,33}\) or by general mechanisms, such as dissipation of proton motive force (PMF), necessary for substrate translocation, rendering it difficult to distinguish between on- and off-target inhibitors of this target.\(^ {34,35}\)

Here, we report a whole-cell assay encompassing a transcriptionally regulated strain of MmpL3, mmpL3-TetON, with two-way regulation of protein expression in Mtb to identify and segregate on-target inhibitors. We first characterized the consequences of regulating MmpL3 on growth, membrane permeability, and susceptibility to known MmpL3 inhibitors and existing antibiotics. Next, we performed a cell-based phenotypic screen utilizing the mmpL3-TetON strain against 220 compounds comprising the GSK-Tres Cantos antmycobacterial set (TB-set) to identify new inhibitors in a cellular context.\(^ {36}\) The mmpL3-TetON strain and wild-type H37Rv (WT) grew with similar kinetics in either the absence or presence of 500 ng/mL of anhydrotetracycline (ATc) (Figure 1A). MmpL3 expression was quantified by LC-MS/MS using MmpL3-specific peptides and normalized to the essential sigma factor SigA of RNAP, which is constitutively expressed during growth and commonly used as reference in expression studies.\(^ {37}\) Compared to WT, mmpL3-TetON with ATc displayed a 717% increase in MmpL3 expression while its cultivation in ATc-free media resulted in an 80% decrease (Figure 1B). Therefore, mmpL3-TetON provides two-way regulation, overexpression, and under-expression of MmpL3, without compromising the growth of Mtb.

Next, we examined the susceptibility of mmpL3-TetON toward the clinical candidate SQ109 (diamine) (Figure 1C) and three other compounds, IC2418 (indolecarboxamide) (Figure 1D), BM212 (pyrrole) (Figure 1E), and AU1235 (adamantyl urea) (Figure 1F), that reduce the growth of Mtb by inhibition of MmpL3. All four compounds were more potent against mmpL3-TetON than WT when both strains were grown without ATc and less potent against mmpL3-TetON when grown with ATc (Figure 1C–F, Table S1). The dose–response profiles thus showed a bidirectional shift in IC\(_{50}\) in response to MmpL3 regulation, suggesting that mmpL3-TetON could be used to identify inhibitors of MmpL3.

### RESULTS

**Consequences of Two-Way Regulation of MmpL3 on Growth, Protein Expression, and Susceptibility to On-Target MmpL3 Inhibitors.** We constructed a transcriptionally regulated MmpL3 strain, mmpL3-TetON, as previously described for trxB.\(^ {36}\) The mmpL3-TetON strain and wild-type H37Rv (WT) grew with similar kinetics in either the absence or presence of 500 ng/mL of anhydrotetracycline (ATc) (Figure 1A). MmpL3 expression was quantified by LC-MS/MS using MmpL3-specific peptides and normalized to the essential sigma factor SigA of RNAP, which is constitutively expressed during growth and commonly used as reference in expression studies.\(^ {37}\) Compared to WT, mmpL3-TetON with ATc displayed a 717% increase in MmpL3 expression while its cultivation in ATc-free media resulted in an 80% decrease (Figure 1B). Therefore, mmpL3-TetON provides two-way regulation, overexpression, and under-expression of MmpL3, without compromising the growth of Mtb.

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Compounds Indirectly Affected by MmpL3 Display a Unidirectional Shift against mmpL3-TetON. Next, we investigated the potency of chemically and mechanistically diverse antibiotics toward WT and mmpL3-TetON. The potency of 22 antibiotics (Figure S1) was similar against mmpL3-TetON and WT when grown in the absence of ATc. This included fluoroquinolones, such as ciprofloxacin, and mycolate synthesis inhibitors, such as isoniazid, that behaved as previously reported. However, when cultivated without ATc, mmpL3-TetON was hyper-sensitized to rifampicin (Figure 2A), clarithromycin (Figure 2B), erythromycin, fidaxomicin (Figure 2C), and fusidic acid (Figure 2D). MmpL3 depletion also increased activity of different β-lactams, cephalexin (Figure 2C), cefadroxil, cefdinir (Figure 2E), ampicillin (Figure 2D), carbencillin (Figure 2H), doripenem (Figure 2E), imipenem, and faropenem (Figure 2F). In contrast to the direct inhibitors of MmpL3 activity, the potency of these compounds did not shift for mmpL3-TetON when grown in the presence of ATc. A unidirectional MIC shift thus distinguished compounds that were affected indirectly by a reduction in MmpL3 expression from direct MmpL3 inhibitors that had displayed a bidirectional MIC shift.

MmpL3 mediates the transport of TMM, which suggests that reducing its expression could alter the fluidity of the mycobacterial cell envelope and modify its permeability. To test this, we performed an ethidium bromide (EtBr)-based uptake assay for WT and mmpL3-TetON. When grown with ATc, EtBr uptake of mmpL3-TetON was similar to that of WT (Figure 2F). However, when grown without ATc, mmpL3-TetON exhibited a 3-fold increase in EtBr uptake relative to the WT. The increased susceptibility that resulted from under-expression of MmpL3 for growth inhibition by mechanistically diverse drugs such as rifampicin, clarithromycin, erythromycin, and fidaxomicin, which are all large (>500 Da) and hydrophobic (log P > 2.5), might thus be due to differences in drug uptake. Conversely, the increased potency of hydrophilic antibiotics such as β-lactams could be governed by defects in outer membrane permeability or by mechanisms not entirely understood.

Figure 2. Silencing of MmpL3 expression alters cell envelope permeability to specific off-target inhibitors. (A–E) Dose–response profiles of mmpL3-TetON (orange) in the absence (open, dashed) and presence (closed, solid) of ATc (500 ng/mL) against high molecular weight antibiotics (A) rifampicin and (B) clarithromycin; and to different classes of (C–E) β-lactams. Data are representative of three experiments; values are averages of three technical replicates and error bars represent the SE of the mean. (F) Impact of MmpL3 depletion on accumulation of EtBr. The strains were incubated with 8 μg/mL of EtBr in PBS supplemented with 0.05% Tyloxapol and 0.4% Glucose. Reads were collected every minute until 60 min at 37 °C. See also Figure S2.

Figure 3. Susceptibility profile of mmpL3-TetON against the on-target hits. (A–C) Impact of selected screening hits on mmpL3-TetON (orange) and WT (black). For mmpL3-TetON, measurements were performed without (open squares, dotted lines) and with (closed squares, solid lines) ATc (500 ng/mL). Data are representative of three biological and two technical replicates. See also Figure S3 and Table S1.
Targeted Whole-Cell Phenotypic Screen to Identify Inhibitors of MmpL3 Function. To investigate the utility of two-way regulation in identifying scaffolds that inhibit mycolate transport, we evaluated mmpL3-TetON and WT against a library of 220 compounds in the GSK Tb-set. Bacteria were grown without ATc and subjected to up to 10 doses of the compounds with concentrations ranging between 100 and 0.005 μM including 1% DMSO (negative control) and 1 μg/mL rifampicin (positive control). The screen presented a Z' value >0.8. Compounds that inhibited the growth of WT by at least 30% at the highest concentration were re-evaluated against WT and mmpL3-TetON with and without ATc. This identified 26 hits (Table S1, Figure S3), which were then assessed for changes in IC_{50}. All 26 compounds displayed a decrease in IC_{50} against the mmpL3-TetON without ATc. Of these, 12 compounds displayed >1.5-fold increase in IC_{50} against mmpL3-TetON with ATc, which were categorized as on-target hits. Six of the on-target hits (denoted with * in Table S1) were excluded from subsequent analyses due to lack of availability and poor solubility. The remaining hits were distributed in three scaffolds including Spiros (Figure 3A and Table S1), oxadiazoles (Figure 3B and Table S1), and THPPs (Figure 3C and Table S1).

Interestingly, we observed inconsistency in phenotype for members of two scaffolds, Spiros (SB-354364, Table S1) and cycloalkanes (GSK2783100A*, Table S1), as neither confirmed to target MmpL3. The singleton hydroxypiperidine (GSK1985270A, Table S1) that seems to target MmpL3 in both Mtb and Mycobacterium abscessus (Mab) displayed no change in potency against the mmpL3-TetON with ATc.

The THPPs identified herein accounted for 42% of the on-target hits with all presenting a bidirectional shift of the highest magnitude of the scaffolds tested. Mutations conferring THPP resistance also map to one target, MmpL3, and THPPs bind MmpL3, rendering it a pivotal target. However, THPPs also interact with an enoyl-coenzyme A hydratase, EchA6, to suppress mycolic acid synthesis, deeming it a second target. We confirmed the stereospecificity of new THPPs for EchA6 (Figure S4) in an intrinsic tryptophan fluorescence binding assay against the purified Mtb-EchA6. As expected, the compounds bound with a K_{D} ranging from 0.62–6.86 μM, similar to the previously reported THPPs, GSK366A (K_{D} = 1.01 ± 0.38 μM) and GSK951A (K_{D} = 0.64 ± 0.16 μM), further supporting EchA6 as a second target.

Partial Expression of MmpL3 Identifies Inhibitors of Cytochrome bc_{1}-aa_{3}. The crystal structure of MmpL3 defined a dedicated H+ translocation channel in the protein that drives the translocation of TMM across to the periplasmic space. Co-crystal structures and molecular docking studies suggest that most MmpL3 inhibitors reduce TMM translocation by binding and blocking the H+ relay channel. This motivated us to further explore the relationship between membrane polarization and MmpL3 activity. We first analyzed the impact of MmpL3 expression on the membrane potential (ΔΨ) using the ΔΨ-sensitive cyanine dye DiOC2(3). Red fluorescence of DiOC2(3) indicates an intact membrane potential, whereas its dissipation, for example, by treatment with the PMF uncoupler CCCP, is accompanied by a shift from red to green fluorescence. DiOC2(3) fluorescence of mmpL3-TetON grown with ATc was similar to that of WT but red-shifted when grown without ATc. This suggested that MmpL3 under-expression led to an increase in ΔΨ, as reported previously, while its overexpression led to a small decrease in ΔΨ (Figure 4A).

RND transporters rely on PMF and PMF-disrupters, such as CCCP, can inhibit the RND transport activity. Molecules that affect the electron transport chain (ETC), which is responsible for generation and maintenance of PMF, could thus represent one class of inhibitors whose potency increases upon MmpL3 under-expression without interacting with MmpL3 directly. We tested the impact of MmpL3 under-expression on ETC inhibitors with Q203 and bedaquiline, that inhibit Mtb’s cytochrome bc_{1}-aa_{3} oxidase and ATP synthase, respectively. Silencing of MmpL3 only had a modest effect on bedaquiline but enhanced the activity of Q203 (Figure 4B,C), which identified inhibitors of cytochrome bc_{1}-aa_{3} oxidase as potential off-target hits.

Furthermore, the hits we classified as indirect inhibitors of MmpL3 included two scaffolds, imidazopyridine amine (IPA) and quinolone (QoA), reported to target cytochrome bc_{1}-aa_{3} oxidase. The cytochrome bc_{1}-aa_{3} oxidase functions in conjunction with cytochrome bd oxidase and inactivation of both leads to rapid killing of Mtb. Accordingly, inhibitors of cytochrome bc_{1}-aa_{3} oxidase are more potent against a knockout of cytochrome bd oxidase (ΔcyaABD) than WT. To
investigate whether the remaining off-target scaffolds included additional cytochrome bc$_{1}$-aa$_{3}$ oxidase inhibitors, we measured their activity against ΔcydABDC. In concordance with previous results, the MIC of bedaquiline against the ΔcydABDC remained relatively unchanged (Figure 5A) whereas the MIC of Q203 decreased substantially (Figure 5B). A sulfanylacetamide, GSK1859936A, demonstrated augmented potency akin to Q203 (Figure 5C), suggesting cytochrome bc$_{1}$-aa$_{3}$ oxidase as the target of GSK1859936A. The potency of scaffolds that inhibit MmpL3 directly remained largely unchanged against the knockout of cytochrome bd oxidase (Figure S5). Deletion of cydABDC too did not change the MIC of thienopyrimidine amines (TPAs), aminothiazoles, or pyridine-2-carboxamides, indicating an alternative mode of growth inhibition by these scaffolds (Figure S6). In contrast, IPAs and QoA were, as expected, more potent against ΔcydABDC, which further validates cytochrome bc$_{1}$-aa$_{3}$ as a target for these scaffolds (Figure S7).

Guanidine-Based CCI7967 Is an MmpL3 Inhibitor. Our screen classified CCI7967, 1-(4-Chlorophenyl)-2-[4-methyl-6-(2-phenylethylamino) pyrimidin-2-yl] guanidine (Figure 6A), as a direct inhibitor of MmpL3. Its IC$_{50}$ was 2.4-fold lower against mmpL3-TetON than WT in the absence of ATc and 4.3-fold higher against mmpL3-TetON than WT in the presence of ATc (Figure 6B and Table S1). To gain further insight into its target, we isolated spontaneous mutants to CCI7967. Approximately 9 × 10$^{-7}$ of the bacteria plated grew on agar plates containing CCI7967 at a concentration of 4-fold the MIC. This suggests that the frequency of resistance against CCI7967 is similar to what has been observed for other......
MmpL3 inhibitors, for which resistance frequencies range between $10^{-7}$ and $10^{-8}$–$10^{-9}$.

Whole-genome sequencing identified two SNPs, T284A and A677T, in MmpL3 that imparted a 5- to 8-fold change in IC$_{50}$ against CCI7967 (Figure 6C). We also assessed the cross-inhibitory activity of CCI7967 toward previously reported MmpL3 resistant mutants isolated against Spiros and THPP compounds to confirm MmpL3 as the target (Figure 6C). The fold changes in IC$_{50}$ were variable, with the S991 SNIP imparting up to 35-fold resistance to CCI7967. This substitution was previously found to confer pan-resistance to several MmpL3 inhibitors.

Previously, a synergistic association of SQ109 with rifampicin and β-lactams was assigned to be a signature of MmpL3 inhibitors. On this basis, we determined if CCI7967 synergizes with rifampicin and β-lactams. The drug combinations were assessed in a checkerboard assay format and the isobolograms were generated by plotting the FIC$_A$ vs FIC$_B$ (Figure 6D–F). As expected, the combination of CCI7967 with cefadroxil (Figure 6C) and rifampicin (Figure 6D) presented FICI $\leq 0.5$ with a convex, inward-bowing isobologram indicative of synergism while no significant interaction of CCI7967 with SQ109 (Figure 6F) was evident, suggestive of a shared target for the two inhibitors. Collectively, these data confirm CCI7967 to be an MmpL3 inhibitor.

**DISCUSSION**

MmpL3 is essential to the integrity of the mycobacterial cell envelope which contributes to the intrinsic resistance of mycobacteria to antimicrobials. To facilitate discovery and development of MmpL3 inhibitors, we developed a simple and robust assay that utilized two-way regulation of MmpL3 expression to select for target-specific compounds. That under-expression can be exploited to identify small-molecules that inhibit a target of interest in Mtb has been demonstrated previously.

However, the two-way system described herein greatly facilitates to distinguish molecules that engage the target of interest directly from those that are potentiated due to indirect effects. Easy to implement in HTS format, when applied against the GSK Tb-set, this methodology validated several of the previously identified MmpL3 inhibitors with a few exceptions, notably, SB-354364 (Spiros), and a closely related compound, GSK1985270A (hydroxyipiperidine) (Figure S3). The two cycloalkanes (GSK2623870A* and GSK2783100A*) also displayed differences in their dependence on MmpL3 expression. Since high vulnerability of MmpL3 can potentially mask secondary effects or targets of the selected hits, it is plausible that the variation observed in these cases could be attributed to scaffolds having secondary targets, as is evident for THPPs and for a few analogues of Spiros.

The likelihood of a compound from a scaffold presenting a bidirectional shift in our assay relies on its affinity toward MmpL3, and when structurally modified could alter this bias in favor of the secondary target rendering it difficult to be identified as an on-target inhibitor. An example of this was seen for THPPs, wherein a point mutation, W133A in EchA6, increased the in vitro MIC and effective dose in mice.

Interestingly, THPP-resistance conferring mutations mapped to the H$^+$ translocation domains of MmpL3, similar to all other MmpL3 inhibitors identified to date, suggesting that increased efflux is not the mechanism of resistance. Therefore, it is likely that MmpL3’s vulnerability coupled with its ability to bind diverse scaffolds and a range of cellular ligands could render it as a sink for most compounds consequently limiting their access to other target(s).

Molecules whose potency increased in response to MmpL3 depletion but did not decrease after MmpL3 overexpression were classified as off-target hits. For several hits, including TPAs (GSK237561A and GSK153890A), aminothiazoles (GSK690382A and GSK445886A), and a pyrrole-2-carboxamide (GSK1750922A), this classification was supported by cross-resistance measurements as IC$_{50}$ values were only affected 0.4- to 5-fold by known resistance mutations in mmpL3 (Table S2). Inhibitors of cytochrome bc$_{2}$-aa$_{3}$ oxidase, including IPA, QoA, and piperazine, and a novel sulfanylamide (GSK1859936A), were frequent off-target hits. Enrichment of these inhibitors in our screen might be related to the repression of genes linked to energy metabolism that has been observed in response to inactivation of MmpL3. The mechanisms underlying other off-target inhibitors likely include increased target access, as seen for rifampicin and β-lactams, and, potentially, inhibition of other steps in or linked to mycolic acid synthesis or transport, a possibility that requires further exploration.

Our assay also identified a novel guanidine, CCI7967, as an MmpL3 inhibitor with the characteristic bidirectional shift. Its on-target activity was further evidenced by resistance mutants against the compound yielding mutations in MmpL3 and cross-resistance to other SNPs in the transporter. These three approaches—under-expression, overexpression, and identification of resistance mutations—helped discern CCI7967 as an on-target inhibitor, which can be confirmed in future studies by monitoring TMM accumulation, or direct interaction with MmpL3. Furthermore, the interaction of CCI7967, similar to previously reported MmpL3 inhibitors, was synergistic with rifampicin and cefadroxil but additive with SQ109. The cell-based assays we developed can thus be used to screen for new small molecules that inhibit the growth of Mtb in an MmpL3-dependent manner and subsequently distinguish those molecules that are likely to target MmpL3 directly from those whose potency is only indirectly affected by MmpL3.

Aside Mtb, MmpL3 is emerging as an important drug-target in other rapidly growing pathogenic nontuberculosis mycobacteria such as Mab. An arising opportunist, Mab is inherently resistant to several of the existing antibiotics which drastically limits the treatment options for infection. At present, the discernment of new MmpL3 inhibitors with activity against Mab relies on phenotypic screens and cross-species inhibition experiments. Since Mab and Mtb are closely related, the two way system described herein could subsequently be adapted in Mab to supplement the ongoing drug discovery efforts.

**METHODS**

**Construction of mmpL3-TetON.** We first generated an mmpL3 merodiploid by integrating the constitutive mmpL3 expression plasmid pGMCS-Ptb38-mmpL3 (Strep) into the attachment site of the phage L5 (attL5) of M. tuberculosis H37Rv. Next, the wild-type (WT) copy of mmpL3 was inactivated by homologous recombination using the knockout construct previously used to generate mmpL3-DUC. Deletion of the WT copy of mmpL3 resulted in H37Rv pGMCS-Ptb38-mmpL3, in which the only copy of mmpL3 is expressed from the attL5 site. The mmpL3-TetON was generated by transforming H37Rv pGMCS-Ptb38-mmpL3 with the mmpL3-TetON plasmid pGMCS-0X-T10-M-P606-
SC-mmpL3 and screening for colonies that were resistant to zeocin and susceptible to streptomycin. Streptomycin sensitivity assured replacement of the constitutive pGMCSPeb38-mmpL3 by pGMCZ-0X-T10-M-P606-SC-mmpL3. Plasmid replacement and regulation of MmpL3 expression was further confirmed by protein quantification and phenotypic assays.

**Plasmids, Bacterial Strains, and General Procedures.** The WT and its derivatives were grown in Middlebrook 7H9 medium supplemented with 0.2% (v/v) glycerol, 0.05% (v/v) tyloxapol, 0.5% (w/v) Bovine Serum Albumin (BSA, Roche), 0.2% (w/v) dextrose, and 0.085% (w/v) NaCl (ADN), or on Middlebrook 7H10 agar supplemented with 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC; BD, Difco) and 0.5% (v/v) glycerol at 37 °C unless otherwise specified. Hygromycin (Roche), streptomycin (Sigma-Aldrich), kanamycin (Sigma-Aldrich), zeocin (Thermo Fisher) and anhydrotetracycline (ATc, Sigma-Aldrich) were used at a concentration of 50, 25, 25, and 25 μg/mL, respectively. For experiments with the regulated strain, the wild-type and mmpL3-TetON strains were cultured until mid-log phase (OD580 0.6–0.8) in the presence of 500 ng/mL ATc at 37 °C. To trigger regulation, cells were harvested, washed, and resuspended to an OD580 ≈ 0.05 in Middlebrook 7H9–ADN supplemented with and without ATc and incubated for up to seven generations (doubling time approximately 24 h) at 37 °C. Protein levels were further altered by rediluting cultures to an OD580 ≈ 0.01 and incubated for a minimum of five generations (doubling time approximately 24 h) under the same growth conditions as mentioned above to perform subsequent experiments. The knockout of cytochrome bd oxidase (ΔcytABDC) and complement was grown as previously described.35

**Identification and Quantitation of MmpL3 by LC-MS/MS.** The transcriptionally regulated strain of mmpL3 and the parental H37Rv were initially grown until mid-log phase in the presence of 500 ng/mL ATc at 37 °C. To trigger regulation, cells were harvested, washed, and resuspended to an OD580 ≈ 0.05 in Middlebrook 7H9–ADN supplemented with and without ATc and incubated for up to seven generations (doubling time approximately 24 h) at 37 °C. Protein levels were further altered by rediluting cultures to an OD580 ≈ 0.01 in same growth conditions and incubated for a minimum of five generations (doubling time approximately 24 h). Bacterial lysates for targeted proteomics were prepared using a previously published protocol.72 Briefly, protein extracts were prepared from 30 mL cultures by bead beating pellets with Lysing Matrix Beads (MP Biomedical) in PBS supplemented with 2% SDS and complete EDTA–pro tease–free inhibitor cocktail (Roche). Lysate protein concentrations were determined using a BCA protein assay kit (Pierce, Thermo Scientific). Lysate corresponding to 50 μg total protein were processed for trypsin digestion and subjected to LC/MS/MS for Parallel Reaction Monitoring (PRM) analysis similar to that published previously.73 Briefly, a 3.0 μL aliquot of trypsin-digested sample was directly injected onto a custom packed 2 cm × 100 μm C18 Magic 5 μm particle trap column. Peptides were then eluted and sprayed from a custom packed emitter (75 μm × 25 cm C18 Magic 3 μm particle) with a linear gradient from 95% solvent A (0.1% formic acid in water) to 35% solvent B (0.1% formic acid in Acetonitrile) in 90 min at a flow rate of 300 nanoliters per minute on a Waters Nano Acuity UPLC system. Data dependent acquisitions were performed on a Q Exactive mass spectrometer (Thermo Scientific) according to an experiment where full MS scans from 300 to 1750 m/z were acquired at a resolution of 70 000 followed by 10 MS/MS scans acquired under Higher-energy C-trap dissociation (HCD) fragmentation at a resolution of 17 500 with an isolation width of 1.6 Da. Six tryptic peptides of MmpL3 (residues 97–113 FQDQHPDQVLGWAGYLR, 163–182 LAGLQPVQAELTGTIAATDQR, 510–523 VLGQNLINPADASK, 744–755 IGLGEHILDPER, 801–829 SSPASPELTPALEATAAAPSGASTTR, and 882–900 STDAA-GDPxAEPTAALPIIR) and four tryptic peptides of SigA (238–251 VALLNAEEVEVL, 410–418 VLEIQYQR, 479–487 FGLTDGQPR, and 488–500 TLEDEIGQYVGVT; all annotations as per http://tuberculist.epfl.ch) were selected to monitor the protein abundance in the samples. Three fragment ion intensities that generated the most robust signals were extracted from each peptide and summed to determine the peptide abundance. Quantification of targeted peptides was accomplished through the Skyline software (University of Washington). The average of peptide abundance from two technical replicates were analyzed for each sample. The relative level of MmpL3 was calculated by normalizing the total transition area of each of the six MmpL3 peptides to that of each of the four SigA peptides. Data represent the mean ± SE obtained from the normalized values.

**Drug Susceptibility Assays.** The compounds used in this study were sourced from Sigma-Aldrich and GlaxoSmithKline (GSK). Bedaquiline, Q203, Pretomanid (PA-824), and BM212 were procured from MedChemExpress. IC2418 and AU1235 were sourced from Mary Jackson, Colorado State University. Compounds had a purity of >98%, as determined by HPLC and 1H NMR analysis and were dissolved and stored in dimethyl sulfoxide for use. Susceptibility assays were performed in black/clear, flat-bottom 384-well microplates (Grenier Bio-One) by dispensing nanoliter volumes of drugs into 2-fold dilutions with a minimum of 10 doses per drug using an automated drug dispenser (D300e Digital Dispenser, HP). The DMSO concentrations in all wells were normalized to 1%, controls included DMSO at 1% and rifampicin at 1 μg/mL for 100% growth and 100% inhibition, respectively. Mid log phase cultures were diluted to an optical density (OD580) ≈ 0.01 in fresh 7H9–ADN media and wells were seeded with 50 μL of bacteria. Inoculated plates were stacked, covered with a sterile lid, wrapped with aluminum foil to prevent evaporation, and allowed to incubate at 37 °C, 5% CO2 at 80% relative humidity for up to 10 days. Growth measurements were recorded at OD580 using a spectrophotometer (SpectraMax M5 or M2e). IC50 values were determined using the variable-slope four-parameter nonlinear least-squares regression model in GraphPad Prism software package (version 8). To determine the MIC of the compound on solid media, 7H10 agar supplemented with 10% OADC and 0.5% glycerol was used to prepare 2-fold serial dilutions of CC17967 at concentrations ranging from 100–0.097 μM. Mid log phase cultures were grown to an OD580 of 0.8–1 and diluted to a final concentration of 106 CFU/mL and 10 μL was spotted onto each dilution. The plates were incubated at 37 °C for up
to 4 weeks. The MIC was defined as the lowest concentration of drug that prevented the growth of Mtb.

**High Throughput Screening.** The pilot screening was performed at GSK, Tres Cantos, Spain using exponentially growing WT and mmpL3-TetON as previously described in Middlebrook 7H9–ADN medium and flat bottom black/clear 384-well plates (Grenier Bio-One) containing 220 compounds of the GSK Tb-set dispensed via Echo acoustic liquid handler. The single cell suspensions were prepared by centrifuging the cultures at 800 rpm for 10 min. The resulting suspension of strains was diluted to an OD$_{600}$ = 0.01 and dispensed onto the compound plates using the Multidrop dispenser (Thermo Fisher) followed by incubation at 37 °C, 5% CO$_2$ at 80% relative humidity for up to 10 days. Rifampicin at 1 μg/mL was used as positive control for inhibition and DMSO was maintained at 1%. The OD$_{600}$ for two treatment groups were read using a SpectraMax MS and the data was analyzed using Activity Base software to get primary hits. Subsequent confirmatory screenings were performed with shortlisted hits in a similar manner as described for drug susceptibility assays. The robustness of data was assessed by Z’ factor with a value of >0.8 as the cut off limit.

**EchA6 Binding Assay.** The recombinant protein, EchA6, was expressed and purified as previously described. Briefly, *Escherichia coli* BL21 (DE3), harboring the pET28a-echA6 vector, were grown in 1 L LB at 37 °C, 180 rpm, to OD$_{600}$ of 0.4–0.6. Recombinant EchA6 expression was induced by the addition of 1 mM Isopropyl β-1-thiogalactopyranoside (IPTG), at 16 °C for 24 h. Following harvesting, the cell pellet was resuspended in 50 mM sodium phosphate, 600 mM sodium chloride and 10 mM imidazole (pH 8) containing a poly histidine tag. The pure protein was subsequently dialyzed by exploiting the vector-encoded chromatography (IMAC) and analyzed in GraphPad Prism 6.

**Membrane Potential Measurements in Intact Mtb.** The effect of over- and under-expression of MmpL3 on transmembrane potential ($\Delta \Psi$) in intact bacilli was determined using fluorescence quenching of the $\Delta \Psi$-sensitive probe 3,3'–diethyloxacarbocyanine iodide (DiOC$_2$(3)) (Thermo Fisher). WT H37Rv and mmpL3-TetON were labeled with 50 μM DiOC$_2$(3) in fresh 7H9–ADN for up to 30 min. Excess DiOC$_2$(3) was washed off and an equal volume of bacteria at an OD$_{600}$ of 0.8 were then aliquoted in black 96-well plates (clear bottom) for reading. Samples were also treated with 500 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP, positive control). The change in fluorescence due to disruption of $\Delta \Psi$ was monitored with a fluorescence spectrophotometer (Spectramax M2e). DiOC$_2$(3) was excited at 485 nm, and emission was measured at 515 and 535 nm. The red/green (610 nm/535 nm) fluorescence intensity ratio was calculated and used to quantify the strength of $\Delta \Psi$.

**Ethidium Bromide Uptake Assay in Microtiter Plates.** Strains were grown to OD$_{600}$ of 0.8–1.0 as described above. Cells were washed once and resuspended in PBS supplemented with 0.02% tyloxapol and adjusted to an OD$_{600}$ of 0.8. Glucose was then added to a final concentration of 0.8% to trigger metabolism and cells were distributed at 100 μL per well in a black 96-well microtiter plate with clear bottoms. Subsequently, an equal volume of ethidium bromide in PBS supplemented with 0.02% tyloxapol and 0.8% glucose was added to a final concentration of 4 μg/mL. The kinetics of fluorescence was measured every minute for 60 min at 37 °C using a fluorescence spectrophotometer (Spectramax M5, excitation at 530 nm, emission at 590 nm). Measurements were performed in triplicates.
Similarly, the FIC for compound B (FIC<sub>B</sub>) was calculated. The FIC Index (FICI) was calculated as FICI = [FICA + FICB]. Synergy was defined by FICI values ≤0.5, antagonism by FICI values >4.0, and no interaction by FICI values from 0.5 to 4.0.

**Quantification and Statistical Analysis.** Graphic data were prepared with GraphPad Prism software and the results were analyzed as stated in the text. Two-tailed unpaired Student’s t test was used for the analysis of differences between two groups. Statistical significance was defined as P < 0.05 unless otherwise stated.

**Data Availability.** Compound structures are available at ChEMBL (https://www.ebi.ac.uk/chembl/) and can be accessed using the doc ID CHEMBL2095176, previously published in Ballell, L. et al., 2013.3

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00675.

Susceptibility profile of under-expressor against drugs of known mechanism of action (Figure S1), large molecular weight antibiotics (Figure S2), structures of hits (Figure S3), binding of EchA6 to the THPPs as identified (Figure S4), scaffolds that do not interfere with Mtb ETC (Figure S5 and S6), scaffolds that interfere with Mtb ETC (Figure S7), susceptibility of mmpL3-TetON to hits (Table S1), cross-resistance of MmpL3 mutants against the compounds of unknown mechanism of action (Table S2), strains and plasmids (Table S3) (PDF)

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#### Notes

The authors declare no competing financial interest.

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### ABBREVIATIONS

Mtb, Mycobacterium tuberculosis; Mab, Mycobacterium abscessus; TB, tuberculosis; WHO, World Health Organization; WCS, whole-cell screens; RND, resistance nodulation and division; TMM, trehalose mono mycolate; THPP, tetrahydropyrazolopyrimidines; Spiros, spirocycles; PMF, proton motive force; GSK, GlaxoSmithKline; TB-set, GlaxoSmithKline-Tres Cantos antimycobacterial set; ATc, anhydrotetracycline; ETC, electron transport chain; IPA, imidazopyridine amine; QoA, quinolone; TPA, thienopyrimidine amine; CCCP, carbonylcyanide m-chlorophenyl hydrazone, 3,3′-diethyloxocarcobycanine iodide; EtBr, ethidium bromide; IPTG, isopropyl β-D-thiogalactopyranoside; EchA6, enoyl coenzyme A hydratase; HTS, high throughput screen; SNPs, single nucleotide polymorphisms; OADC, oleic acid albumin dextrose catalase; ADN, albumin dextrose sodium chloride; IMAC, immobilized metal affinity chromatography; SE, standard error of the mean; FIC, fractional inhibitory concentration.


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