Common biological features of *Mycobacterium tuberculosis* MmpL3 inhibitors

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Abstract

MmpL3 is a promising new target for antitubercular drugs, but the microbiological properties of MmpL3 inhibitors are not fully understood. We compared the activity and mode of action of 11 structurally diverse compound series that target MmpL3. We confirmed activity was via MmpL3 using strains with differential expression of MmpL3. MmpL3 inhibitors had potent activity against replicating *M. tuberculosis*, with increased activity against intramacrophage bacilli and were rapidly bactericidal. MmpL3 inhibition induced cell wall stress concomitantly with a boost in ATP levels in *M. tuberculosis*. Mutation in MmpL3 conferred resistance to all series at different levels. Molecules did not negatively impact membrane potential, pH homeostasis or induce reactive oxygen species and were inactive against starved bacilli. Our study revealed common features related to the chemical inhibition of MmpL3, enabling the identification of off-target effects and highlighting the potential of such compounds as future drug candidates.

Introduction

Tuberculosis (TB) remains a leading cause of mortality due to a single infectious disease, causing 10.8 million active infections and 1.25 million deaths in 2023[1]. Current treatment regimens for TB infections are lengthy, requiring four antibiotics taken for four to six months. Drug-resistant infections, accounting for 400,000 infections in 2023[1], require an extended treatment time of up to 24 months with second line drugs. Many of the existing TB drugs are associated with adverse effects including visual impairment, hepatoxicity, neuropathy and QTc prolongation[2–4]. To improve treatment outcomes, there is an urgent need for more effective drugs that shorten treatment times and with fewer adverse effects. Identifying compounds with new targets is key to this goal.

Mycobacterium tuberculosis, the causative agent of TB, has a thick cell wall vital for survival in the host environment. The cell wall consists of a highly hydrophobic layer of mycolic acids linked to arabinogalactan which form an impermeable barrier to many antibiotics and is vital for adaptation to the host environment[5]. Preventing *M. tuberculosis* from maintaining its cell wall by treating with cell wall synthesis inhibitors, isoniazid and ethambutol[6–8], has already proven effective in current TB treatment regimens. As a result, there is great interest in the discovery of compounds with other cell wall targets, such as MmpL3. MmpL3 is the sole transporter of mycolic acids in *M. tuberculosis*. It uses the proton motive force (PMF) to transfer TMM (trehalose monomycolate) across the inner membrane to the periplasmic space where it is incorporated into the mycolic acid layer by the Ag85 complex[9–11].

MmpL3 is an attractive drug target since it is unique to *Mycobacteria* sp., is required for survival in macrophages[11,12], for virulence and persistence in murine models of infection[13] and its inhibition results in rapid cell death[11,13–15]. Numerous chemical scaffolds targeting MmpL3 have been described but none have reached approval for clinical use[16]. SQ109 is the furthest in development and reached clinical trials[17–19] but may not be suitable for clinical use and likely has additional targets[20–22].

Studies into the activity and mode of action of MmpL3 inhibitors have been confounded by the off-target effects of some of the most well-studied candidates. For example, SQ109 and BM212 appear to disrupt the PMF[20,21], while others do not. SQ109 is active against *M. tuberculosis* in low oxygen whereas AU1235 and THPP-2 are inactive[20]. In addition, the fact that molecules have been studied in different laboratories under different experimental conditions complicates a direct comparison of activity. Understanding the biology of MmpL3 inhibitors is important in considering how drugs targeting this protein could be used in a multi-drug treatment regimen.

To understand the microbiological properties of MmpL3 inhibitors, we collected a set of structurally diverse compounds that target MmpL3 and simultaneously compared their activity against *M. tuberculosis*, their mechanism of action and interaction with MmpL3 mutant strains. Our study revealed a common set of features across MmpL3 inhibitors, some of which make these compounds promising drug candidates.

Methods

M. tuberculosis culture

Unless otherwise stated, experiments were performed using *M. tuberculosis* H37Rv-LP (ATCC 25618). *M. tuberculosis* strains were cultured in Middlebrook 7H9 medium plus 10 % v/v Middlebrook OADC supplement and 0.05 % w/v Tween 80 and incubated at 37 °C. For starvation, log phase bacteria were harvested and resuspended in PBS plus 0.05 w/v % Tyloxapol and incubated for 7d.

Determination of minimum inhibitory concentration (MIC)

M. tuberculosis was grown to log phase and inoculated to a final OD_{590} of 0.02 in 96-well plates containing compounds as two-fold dilutions. Plates were incubated for 5 days at 37 °C. OD_{590} was read using a Synergy H4 plate reader. Dose response curves were fit using the variable Hill slope model from which IC_{50} and IC_{90} values, the concentration at which 50 % or 90 % growth inhibition occurred respectively, were determined.

Determination of activity using recombinant strains

The assay was performed as previously described[23]. Briefly, the *mmpL3-TetON* strain was grown in 7H9 medium supplemented with 25 µg/mL kanamycin, 25 µg/mL zeocin +/- 500 ng/mL anhydrotetracycline (ATc). Cultures were diluted to OD_{580} of 0.01 in medium +/- ATc and 50 µL was dispensed into 384-well plates containing compound. Plates were incubated at 37 °C with 5 % CO_2. OD_{580} was read after 14 d incubation.

Determination of activity against intracellular bacilli

THP-1 cells were obtained from ATCC (TIB-202) and maintained in RPMI-1640 medium with 10 % FBS and incubated at 37 °C, 5 % CO_2. THP-1 cells were differentiated by treatment with 80 nM PMA for 24 hours prior to overnight infection with an M. *tuberculosis* H37Rv-LP strain constitutively expressing luciferase from the pMV306hsp+LuxG13 plasmid[24] at a multiplicity of infection (MOI) of 1:1. Infected cells were harvested using Accumax™ solution, resuspended in fresh cRPMI and inoculated to a final density of 9 x10^5 cells/mL in 96-well plates containing compounds. Plates were incubated at 37 °C and 5

96 % CO_2 for 72 hours. Luminescence was read using a Synergy H4 plate reader. Dose response curves were
97 fit using the variable Hill slope model and the IC_{50} was determined.

98 **Determination of minimum bactericidal concentration (MBC)**

99 *M. tuberculosis* H37Rv-LP was grown to log phase and inoculated to a final OD_{590} of 0.02 in 96-well
100 plates containing compounds as two-fold dilutions. Plates were incubated at 37 °C. Samples were taken 0,
101 7 and 14 days, spotted onto 7H10 agar plates, and incubated at 37 °C for 2 weeks. The MBC (minimum
102 bactericidal concentration) was determined visually as the lowest concentration at which no growth was
103 observed.

104 **Determination of intracellular ATP levels**

105 *M. tuberculosis* H37Rv-LP was grown to late log phase and inoculated at OD_{590} 0.04 in 96-well plates
106 containing compounds as two-fold dilutions. Plates were incubated at 37 °C for 24 hours. ATP was
107 quantified by adding 50 µL BacTiter-Glo™ reagent per well, incubating for 10 min and reading
108 luminescence using a Synergy H4 plate reader. Kanamycin and Q203 were used as negative and positive
109 controls, respectively.

110 **Induction of cell wall stress**

111 The P_{iniB} reporter assay was adapted from Alland *et al.* 2000[25]. The *M. tuberculosis* P_{iniB}-Lux strain, in
112 which luciferase expression is controlled by the *iniB* promoter[26], was grown in GAST-Fe medium and
113 inoculated to a final OD_{590} of 0.02 in 96-well plates containing compounds as two-fold dilutions. Plates
114 were incubated at 37 °C for 72 hours. 100 µL of substrate solution (28 µg/mL D-luciferin, 50 mM DTT in
115 1 M HEPES buffer) was added to each well and plates were incubated for 25 min at RT. Luminescence
116 was read using a Synergy H4 plate reader. Ethambutol was used as a positive control.

117 **Induction of reactive oxygen species (ROS)**

118 ROS was measured as previously described[27]. Briefly, *M. tuberculosis* H37Rv-LP was grown to an OD_{590}
119 of 1, resuspended in 7H9-Tw + 40 µM 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) and incubated
120 at 37 °C for 30 min. Cells were washed, resuspended in fresh 7H9-Tw medium and inoculated to a final
121 OD_{590} of 0.5 in 96-well plates containing compound. Plates were incubated at 37 °C for 90 mins and
122 fluorescence at Ex485/Em535 nm was measured using a Synergy H4 plate reader. Econazole was used as
123 a positive control.

124 **Determination of membrane potential**

M. tuberculosis H37Rv-LP was grown to late log phase and adjusted to an OD_{590} of 1 in fresh medium. 3,3'-diethyloxacarbocyanine iodide ($DiOC_2$) was added to a final concentration of 15 µM and incubated at RT for 20 min. Cells were harvested, resuspended in fresh medium and inoculated to a final OD_{590} of 0.5 in 96-well plates containing compounds. Plates were incubated at 37 °C for 30 min. Fluorescence was read using an H4 Synergy plate reader at Ex488/Em530 and Ex488/Em610 nm. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a positive control.

Determination of intracellular pH homeostasis

Intracellular pH was measured as previously described[28]. Briefly, *M. tuberculosis* H37Rv-LP expressing a pH-responsive ratiometric GFP (rGFP) from the pHLUOR plasmid[29] was grown to late log phase, washed and resuspended in phosphocitrate buffer pH 4.5 (0.0896 M Na_2HPO_4, 0.0552 M citric acid, 0.05 % tyloxapol). The culture was inoculated to a final OD_{590} of 0.3 into 96-well assay plates containing compounds in two-fold dilutions. Plates were incubated at 37 °C for 48 hours. Fluorescence was measured using an H4 Synergy plate reader at Ex395/Em510 nm and Ex475/Em510 nm. Monensin was used as a control. A standard curve was generated using cell-free extracts as follows: *M. tuberculosis* H37Rv-LP rGFP was lysed using a bead beater then passed through a 0.2 micron filter to obtain cell-free extracts. Cell-free extracts (10 µg/mL protein) were adjusted to pH 5.5 to 8.5 and fluorescence measured to generate a ratio: the standard curve was fit using a four-parameter logistic model.

Results

Compound selection

The goal of our study was to understand the microbiological effects of chemical MmpL3 inhibition in *M. tuberculosis*. To avoid identifying effects related to specific chemical scaffolds, we included 11 chemically diverse compound series in our study (Figure 1). All 11 series originated from phenotypic hits identified in whole cell *M. tuberculosis* screens of chemical libraries (unpublished data) except for the pyrazole series which were derived from the previously described BM212 scaffold to improve its drug-like properties[30]. The selected series encompass varying stages of drug discovery in which activity against *M. tuberculosis* and drug-like properties are optimized. The DDU01, DDU02, DDU03, DDU04, and DDU05 series were early in discovery (hit assessment) in which some structure-activity-relationship exploration had been performed. The indolecarboxamide (ICA) series were the furthest progressed (pre-clinical candidate declaration) of the 11 series; activity and drug-like properties have been well-optimized, and compounds have proven efficacy in mouse models of infection[31]. The remaining series,

156 pyrazole, ARI, 7-aza, oxazole, thiazole, were in mid-stage discovery (hit-to-lead or lead optimization).
157 Two to three compounds representing each of the 11 series were selected for our study (Figure 1;
158 compounds **1-32**). AU1235 was included as a reference since it is a well characterized MmpL3 inhibitor
159 with no known secondary targets[10,14].

160 **Compound series target MmpL3**

161 To confirm MmpL3 as the target, compound activity was measured in *M. tuberculosis* strains with altered
162 MmpL3 expression. The *mmpL3-TetON* strain was used in which MmpL3 expression is regulated by
163 anhydrotetracycline[23] (ATc). In the absence of ATc, expression of MmpL3 protein is decreased ~80% and
164 the *mmpL3-TetON* strain is more susceptible to MmpL3 inhibitors such as AU1235[23] (Figure 2). Similarly,
165 compounds across all 11 series were two- to six-fold more active against the *mmpL3-TetON* strain in the
166 absence of ATc relative to the wild-type H37Rv (Figure 2). In the presence of ATc, MmpL3 expression in
167 the *mmpL3-TetON* strain is increased by more than 7-fold[23]. Interestingly, MmpL3 overexpression
168 conferred resistance to only a subset of the MmpL3 inhibitor compounds in our study and did not confer
169 resistance to AU1235, a well-established MmpL3 inhibitor (Figure 2). Overall, altered activity against
170 strains with differential MmpL3 expression is consistent with MmpL3 as the target of our compound
171 series. Ethambutol was used as a negative control since MmpL3 under expression does not affect
172 sensitivity[23] (Figure 2).

173 **MmpL3 mutations confer resistance**

174 Mutations in the *M. tuberculosis mmpL3* gene confer resistance to a broad range of MmpL3 inhibitors[14,32–
175 34]. To further confirm the target, we looked for resistance using an *M. tuberculosis* MmpL3 mutant strain,
176 RM301 ($MmpL3_{F255L,V646M,F644I}$). This strain was generated via multiple rounds of selection for resistance
177 to different MmpL3 inhibitors and has been shown to confer resistance to structurally diverse MmpL3
178 inhibitor scaffolds[34]. The RM301 strain was >4-fold more resistant to most of our compounds relative to
179 wild-type H37Rv-LP (25 out of 32 compounds; Table 2), consistent with MmpL3 being the primary
180 target. RM301 was most resistant to the 7-aza series; activity against compound **1** was reduced >1639-
181 fold relative to wild-type (Table 2). Of note, the RM301 strain conferred varying levels of resistance
182 between the series, suggesting different interactions or binding affinities with MmpL3 (Table 2).
183 However, mutations in the RM301 strain did not confer resistance to pyrazole or DDU05 series
184 compounds, therefore we selected compound **26** and tested its activity against another MmpL3 mutant
185 strain, $MmpL3_{S591I.}$ This strain was 23-fold more resistant to compound **26** relative to wild-type (Table 2)
186 suggesting the pyrazoles may have a different mode of MmpL3 binding to the other series.

187 **MmpL3 inhibitors inhibit growth of *M. tuberculosis***

188 We measured the MICs (minimum inhibitory concentration) of our compounds against *M. tuberculosis*
189 H37Rv-LP after five days in standard growth medium. All compounds inhibited growth of *M.*
190 *tuberculosis*, some with excellent potency ($IC_{90} < 1$ μM) (Table 1). Compounds **7**, **8** and **9** from the ICA
191 series were most active with an IC_{90} of 0.024, 0.029 and 0.071 μM respectively (Table 1). Compounds
192 from the DDU02, DDU03 and DDU04 series were the least potent; compound **10** had the highest IC_{90} of
193 32 μM (Table 1). In general, compound activity correlated with stage in discovery as compounds in later
194 stages of discovery were more potent than those in early discovery.

195 **MmpL3 inhibitors have enhanced activity against intracellular bacteria**

196 We measured the activity of compounds against intramacrophage *M. tuberculosis.* Differentiated THP-1
197 cells were infected with *M. tuberculosis* H37Rv-LP and exposed to compound for three days. We found
198 that all compounds across all 11 series, as well as AU1235, strongly inhibited growth of intramacrophage
199 *M. tuberculosis* (Table 1). As with extracellular activity, compound activity correlated with stage in
200 discovery. The ICA series was most potent; the intramacrophage IC_{50} for compounds **7**, **8** and **9** was
201 0.063, 0.0060 and 0.0094 μM respectively (Table 1). Compound **19** from the DDU05 series was least
202 potent with an intramacrophage IC_{50} of 12 μM (Table 1). Comparison of intramacrophage and
203 extracellular activity revealed that most compounds, including AU1235, were up to 10-fold more active
204 against intramacrophage *M. tuberculosis* (Figure 3). The increased potency against intramacrophage *M.*
205 *tuberculosis* was observed across chemically distinct compound series so is likely a feature of MmpL3
206 inhibition rather than an effect related to a particular chemical structure.

207 **MmpL3 inhibitors are bactericidal against replicating *M. tuberculosis***

208 We next compared the bactericidal activity of compounds. The minimum bactericidal concentration
209 (MBC) was determined after 14 days exposure to compound in standard growth medium in which the
210 bacilli are in a replicating state. Almost all compounds were bactericidal against *M. tuberculosis* in this
211 condition, a number of which were bactericidal at sub-micromolar concentrations (Table 1). Compounds
212 from the Thiazole and ICA series had most potent bactericidal activity with an MBC less than 0.2 μM (the
213 lowest concentration tested) (Table 1). DDU05 series compound **21** was the only compound for which no
214 bactericidal activity against replicating bacteria was observed up to 100 μM (the highest concentration
215 tested) (Table 1). Across all series, the concentration required for bactericidal activity (MBC) was very
216 close to the inhibitory concentration (IC_{90}; Table 1). Such potent bactericidal activity is an unusual but
217 promising property for antimicrobial compounds.

218 Since *M. tuberculosis* can also exist in a non-replicating state in the host, we looked for bactericidal
219 activity against *M. tuberculosis* cells starved for seven days prior to compound exposure. All compounds,

220 including AU1235, had little or no bactericidal activity against starved cells at the concentrations tested
221 (up to 100 μM; Table 1). Thus, in contrast be previous studies using SQ109[20], we found that MmpL3
222 inhibitors are not active against bacteria in a non-replicating state. This was not surprising since synthesis
223 of the mycolic acid layer, and therefore mycolic acid export, is less important in cells that are not
224 growing. However, the different methods used to induce a non-replicating state (low oxygen compared to
225 nutrient starvation) between studies should be considered.

226 **MmpL3 inhibitors induce cell wall stress**

227 To confirm the mode of action of MmpL3 inhibitors, we ran all compounds through a series of assays
228 testing effects on different biological pathways. We first measured induction of cell wall stress using an
229 *M. tuberculosis* reporter strain in which luciferase expression is controlled by the *iniB* promoter which is
230 induced by cell wall stress[26]. As anticipated for inhibitors of mycolic acid transport, all compounds
231 induced the P_{iniB} reporter indicating cell wall stress (Figure 4). Test compounds induced the reporter at
232 similar levels to ethambutol, an inhibitor of cell wall arabinogalactan synthesis[7,8]. Reporter signal was
233 strongest around the IC_{50} for each compound linking compound activity to the induction of cell wall
234 stress.

235 **MmpL3 inhibitors induce an ATP boost**

236 We measured intracellular ATP following compound exposure. All compounds caused a substantial
237 increase in ATP levels in our assay (Figure 5). This increase occurred at the concentration required to
238 inhibit bacterial growth, suggesting that chemical MmpL3 inhibition induces an ATP burst in *M.*
239 *tuberculosis.* Q203, a QcrB inhibitor[35], was used as a control for ATP depletion and demonstrated the
240 expected decrease in ATP levels at subinhibitory concentrations. Kanamycin (negative control) lead to a
241 drop in ATP levels concomitant with decreased growth.

242 This ATP boost in response to MmpL3 inhibition has not been previously described in *M. tuberculosis*.
243 However, our finding is consistent with studies in other *Mycobacteria* sp., such as *Mycobacterium*
244 *abscessus* and *Mycobacterium bovis* BCG, where treatment with cell wall inhibitors lead to increased ATP
245 concentrations[36,37], suggesting the ATP boost is a general response to cell wall perturbations. Further work
246 is required to understand the mechanistic link between cell wall inhibition and increased ATP generation
247 in *Mycobacteria* sp.

248 **MmpL3 inhibitors to do not affect ROS, membrane potential or pH homeostasis**

249 Compounds were also tested for effects on reactive oxygen species (ROS) generation, dissipation of
250 membrane potential and disruption of intracellular pH. ROS were quantified using 2′,7′-

Dichlorofluorescin diacetate (DCFDA), an oxidation-sensitive fluorescent dye with econazole used as a positive control[27]. Changes in membrane potential were detected using the fluorescent probe 3,3'-Diethyloxacarbocyanine iodide (DiOC2(3)) with monensin, an ionophore, as a positive control. The ability to maintain intracellular pH was tested using *M. tuberculosis* expressing a pH-dependent ratiometric GFP[28]. In general, none of the compounds induced ROS, or disrupted membrane potential or pH homeostasis (Figure 6; Figures S1 to S3). The exceptions were compound **24** from the oxazole series and compound **20** from the DDU05 series which induced intracellular alkalinization (Figure 6, Figure S2), suggesting these molecules have a secondary target. Our finding that structurally diverse MmpL3 inhibitors do not dissipate membrane potential suggest that the effect SQ109 has on membrane potential[20,21] is structure specific, perhaps as a consequence of a second target rather than MmpL3 inhibition.

Taken together, our assays confirmed the mode of action of MmpL3 inhibitors, which induced cell wall stress and increased ATP levels. Changes in membrane potential, pH homeostasis and ROS are not a general feature of MmpL3 inhibition, but rather off-target effects related to specific chemical scaffolds.

Discussion

We performed a systematic comparison of 11 structurally diverse MmpL3 inhibitor series in which 32 representative compounds were run in parallel through a set of biological assays aimed at increasing our understanding of activity against *M. tuberculosis*, mode of action and interaction with MmpL3 mutant strains. AU1235 was chosen as a reference for the study since there is no evidence for off-target activity, to our knowledge.

We confirmed MmpL3 as the target of our compounds using MmpL3 mutant strains. Most MmpL3 inhibitors, including AU1235, SQ109 and NITD-349, bind the same pocket site and induce a conformation change in the MmpL3 protein structure that disrupts two Asp-Try pairs required for proton transport[38]. The same mutations that conferred resistance to AU1235 ($MmpL3_{F255L,V646M,F644I}$ and $MmpL3_{S591I}$), also conferred resistance to our compounds suggesting a similar mode of binding to MmpL3. The fact that MmpL3 mutants conferred different fold-change in resistance to each compound series could suggest different binding affinities to MmpL3. The specificity of resistance to certain compounds series in our study also suggests different modes of binding within the drug pocket. Further understanding the global landscape of MmpL3 mutations and how they relate to resistance will be key to predicting clinical resistance to MmpL3 inhibitors in future treatment regimens.

Compounds from all 11 series behaved similarly to AU1235, highlighting a set of common biological features of MmpL3 inhibitors: a) potent growth inhibition of *M. tuberculosis*; b) bactericidal activity against replicating bacilli; c) increased potency against intramacrophage *M. tuberculosis*; d) induction of cell wall stress; and e) an ATP boost. The identification of core biological features of MmpL3 inhibitors in this study has reinforced the potential of such compounds as potential antitubercular drugs. Many compounds had excellent bactericidal activity and inhibitory activity against intramacrophage *M. tuberculosis*. The contribution of these properties to treatment shortening warrants further study. It will also aid the identification of off-target activity in future drug discovery efforts and inform how such compounds may be used in combinatorial treatment regimens

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References

1. *Global Tuberculosis Report 2024*. (World Health Organization, Geneva, 2024).

2. Forget, E. J. & Menzies, D. Adverse reactions to first-line antituberculosis drugs. *Expert Opin. Drug Saf.* **5**, 231–249 (2006).

3. Ezer, N., Benedetti, A., Darvish-Zargar, M. & Menzies, D. Incidence of ethambutol-related visual impairment during treatment of active tuberculosis. *Int. J. Tuberc. Lung Dis.* **17**, 447–455 (2013).

4. Guglielmetti, L. *et al.* Long-term outcome and safety of prolonged bedaquiline treatment for multidrug-resistant tuberculosis. *Eur. Respir. J.* **49**, 1601799 (2017).

5. Dulberger, C. L., Rubin, E. J. & Boutte, C. C. The mycobacterial cell envelope — a moving target. *Nat. Rev. Microbiol.* **18**, 47–59 (2020).

6. Banerjee, A. *et al.* inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. *Science* **263**, 227–230 (1994).

7. Goude, R., Amin, A. G., Chatterjee, D. & Parish, T. The Arabinosyltransferase EmbC Is Inhibited by Ethambutol in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **53**, 4138–4146 (2009).

8. Telenti, A. *et al.* The emb operon, a gene cluster of Mycobacterium tuberculosis involved in resistance to ethambutol. *Nat. Med.* **3**, 567–570 (1997).

9. Xu, Z., Meshcheryakov, V. A., Poce, G. & Chng, S.-S. MmpL3 is the flippase for mycolic acids in mycobacteria. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 7993–7998 (2017).

10. Stevens, C. M. *et al.* Proton transfer activity of the reconstituted Mycobacterium tuberculosis MmpL3 is modulated by substrate mimics and inhibitors. **119**, (2022).

11. Degiacomi, G. *et al.* Essentiality of mmpL3 and impact of its silencing on Mycobacterium tuberculosis gene expression. *Sci. Rep.* **7**, 43495 (2017).

12. Cheung, C.-Y., McNeil, M. B. & Cook, G. M. Utilization of CRISPR interference to investigate the contribution of genes to pathogenesis in a macrophage model of Mycobacterium tuberculosis infection.

13. Li, W. *et al.* Therapeutic Potential of the Mycobacterium tuberculosis Mycolic Acid Transporter, MmpL3. *Antimicrob. Agents Chemother.* **60**, (2016).

14. Grzegorzewicz, A. E. *et al.* Inhibition of mycolic acid transport across the Mycobacterium tuberculosis plasma membrane. *Nat. Chem. Biol.* **8**, 334–341 (2012).

15. McNeil, M. B. & Cook, G. M. Utilization of CRISPR Interference To Validate MmpL3 as a Drug Target in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **63**, e00629-19 (2019).

16. North, E. J., Schwartz, C. P., Zgurskaya, H. I. & Jackson, M. Recent advances in mycobacterial membrane protein large 3 inhibitor drug design for mycobacterial infections. *Expert Opin. Drug Discov.* **18**, 707–724 (2023).

17. Tahlan, K. *et al.* SQ109 Targets MmpL3, a Membrane Transporter of Trehalose Monomycolate Involved in Mycolic Acid Donation to the Cell Wall Core of Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* **56**, 1797–1809 (2012).

18. Heinrich, N. *et al.* Early phase evaluation of SQ109 alone and in combination with rifampicin in pulmonary TB patients. *J. Antimicrob. Chemother.* **70**, 1558–1566 (2015).

19. Boeree, M. J. *et al.* High-dose rifampicin, moxifloxacin, and SQ109 for treating tuberculosis: a multi-arm, multi-stage randomised controlled trial. *Lancet Infect. Dis.* **17**, 39–49 (2017).

20. Li, W. *et al.* Novel Insights into the Mechanism of Inhibition of MmpL3, a Target of Multiple Pharmacophores in Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* **58**, 6413–6423 (2014).

21. Feng, X. *et al.* Antiinfectives targeting enzymes and the proton motive force. *Proc. Natl. Acad. Sci.* **112**, (2015).

22. Malwal, S. R. *et al.* Investigation into the Mechanism of Action of the Tuberculosis Drug Candidate SQ109 and Its Metabolites and Analogues in Mycobacteria. *J. Med. Chem.* **66**, 7553–7569 (2023).

23. Grover, S. *et al.* Two-Way Regulation of MmpL3 Expression Identifies and Validates Inhibitors of MmpL3 Function in *Mycobacterium tuberculosis*. *ACS Infect. Dis.* **7**, 141–152 (2021).

24. Andreu, N. *et al.* Optimisation of Bioluminescent Reporters for Use with Mycobacteria. *PLoS ONE* **5**, e10777 (2010).

25. Alland, D., Steyn, A. J., Weisbrod, T., Aldrich, K. & Jr, W. R. J. Characterization of the Mycobacterium tuberculosis iniBAC Promoter, a Promoter That Responds to Cell Wall Biosynthesis Inhibition. *J BACTERIOL* **182**, (2000).

26. Naran, K. *et al.* Bioluminescent Reporters for Rapid Mechanism of Action Assessment in Tuberculosis Drug Discovery. *Antimicrob. Agents Chemother.* **60**, 6748–6757 (2016).

27. Howell Wescott, H. A., Roberts, D. M., Allebach, C. L., Kokoczka, R. & Parish, T. Imidazoles Induce Reactive Oxygen Species in *Mycobacterium tuberculosis* Which Is Not Associated with Cell Death. *ACS Omega* **2**, 41–51 (2017).

28. Early, J. *et al.* Identification of Compounds with pH-Dependent Bactericidal Activity against Mycobacterium tuberculosis. *ACS Infect. Dis.* (2019).

29. Darby, C. M. *et al.* Whole Cell Screen for Inhibitors of pH Homeostasis in Mycobacterium tuberculosis. *PLoS ONE* **8**, e68942 (2013).

30. Poce, G. *et al.* Novel Pyrazole-Containing Compounds Active against *Mycobacterium tuberculosis*. *ACS Med. Chem. Lett.* **10**, 1423–1429 (2019).

31. Stec, J. *et al.* Indole-2-carboxamide-based MmpL3 Inhibitors Show Exceptional Antitubercular Activity in an Animal Model of Tuberculosis Infection. *J. Med. Chem.* **59**, 6232–6247 (2016).

32. Remuiñán, M. J. *et al.* Tetrahydropyrazolo[1,5-a]Pyrimidine-3-Carboxamide and N-Benzyl-6′,7′-Dihydrospiro[Piperidine-4,4′-Thieno[3,2-c]Pyran] Analogues with Bactericidal Efficacy against Mycobacterium tuberculosis Targeting MmpL3. *PLoS ONE* **8**, e60933 (2013).

33. Li, W. *et al.* Direct Inhibition of MmpL3 by Novel Antitubercular Compounds. *ACS Infect. Dis.* **5**, 1001–1012 (2019).

34. McNeil, M. B. *et al.* Multiple Mutations in Mycobacterium tuberculosis MmpL3 Increase Resistance to MmpL3 Inhibitors. *mSphere* **5**, e00985-20 (2020).

35. Pethe, K. *et al.* Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. *Nat. Med.* **19**, 1157–1160 (2013).

36. Shetty, A. & Dick, T. Mycobacterial Cell Wall Synthesis Inhibitors Cause Lethal ATP Burst. *Front. Microbiol.* **9**, 1898 (2018).

37. Lindman, M. & Dick, T. Bedaquiline Eliminates Bactericidal Activity of β-Lactams against Mycobacterium abscessus. *Antimicrob. Agents Chemother.* **63**, e00827-19 (2019).

38. Zhang, B. *et al.* Crystal Structures of Membrane Transporter MmpL3, an Anti-TB Drug Target. *Cell* **176**, 636-648.e13 (2019).

Table 1. Activity of compounds against *M. tuberculosis*. Inhibition of *M. tuberculosis* H37Rv-LP growth was measured after 5 days compound exposure in axenic culture (extracellular) or infected THP-1 cells (intramacrophage). Bactericidal activity of compounds against *M. tuberculosis* H37Rv-LP under replicating conditions (medium) and non-replicating conditions (starvation) after 14 days exposure. Values show the geometric mean of at least two independent experiments to 2 s.f.

Series	Compound	Extracellular		Intramacrophage IC_{50} (µM)	MBC (µM)	
		IC_{90} (µM)	IC_{50} (µM)		Medium	Starvation
7-aza	**1**	0.061	0.050	0.014	<0.2	100
	2	0.14	0.11	0.024	<0.39	>100
	3	0.17	0.061	0.022	<0.2	25
DDU01	**4**	2.0	1.2	0.62	2.4	>100
	5	5.5	4.3	6.2	9.4	>100
	6	0.19	0.094	0.057	<0.39	>100
ICA	**7**	0.024	0.020	0.016	<0.2	>100
	8	0.029	0.017	0.0060	<0.2	>100
	9	0.071	0.038	0.0094	<0.2	>75
DDU02	**10**	32	10	6.0	25	>100
	11	8.6	6.6	4.5	13	>100
	12	13	5.7	1.2	9.4	100
DDU03	**13**	11	9.5	2.0	13	75
	14	8.3	4.2	3.2	7.8	>100
	15	20	9.2	5.7	27	>100
DDU04	**16**	12	6.0	3.4	6.3	25
	17	3.0	1.9	2.5	3.1	50
	18	15	7.6	4.8	13	>100
DDU05	**19**	8.9	4.1	12	38	>100
	20	6.1	2.1	1.9	9.4	>100
	21	4.8	1.3	3.9	>100	>100
Oxazole	**22**	0.48	0.31	0.060	<0.39	>100
	23	0.078	0.061	0.017	<0.2	>100
	24	0.62	0.46	0.062	1.2	>75
Pyrazole	**25**	0.27	0.12	0.024	<0.39	100
	26	2.3	1.1	0.27	1.6	>100
ARI	**27**	0.58	0.31	0.063	0.78	>100
	28	1.1	0.65	0.24	1.6	>100
	29	2.3	1.0	0.19	3.1	>100
Thiazole	**30**	0.18	0.044	0.019	<0.2	>100
	31	0.19	0.081	0.051	<0.2	>100
	32	0.088	0.053	0.0057	<0.2	>100
AU1235	-	0.22	0.13	0.063	0.59	>100

Table 2. Compound activity against *M. tuberculosis* MmpL3 mutant strains. Inhibition of growth was measured after 5 days exposure to compound. IC_{90} values show the geometric mean of at least two independent experiments to 2 s.f. Fold change was calculated relative to WT IC_{90}.

Series	Compound	WT IC_{90} (µM)	$MmpL3_{F255L,V646M,F644I}$		$MmpL3_{S591I}$	
			IC_{90} (µM)	Fold change	IC_{90} (µM)	Fold change
7-aza	**1**	0.061	>100	>1639	-	-
	2	0.14	>13	>93	-	-
	3	0.17	>50	>294	-	-
DDU01	**4**	2.0	>20	>10	-	-
	5	5.5	>100	>18	-	-
	6	0.19	>100	>526	-	-
ICA	**7**	0.024	1.3	54	-	-
	8	0.029	>2.5	>86	-	-
	9	0.071	>5	>70	-	-
DDU02	**10**	32	>100	>3	-	-
	11	8.6	>100	>11	-	-
	12	13	>95	>7	-	-
DDU03	**13**	11	>20	>1.8	-	-
	14	8.3	>100	>12	-	-
	15	20	>100	>5	-	-
DDU04	**16**	12	>20	>1.7	-	-
	17	3.0	>20	>6.7	-	-
	18	15	>100	>6.7	-	-
DDU05	**19**	8.9	9.6	1.1	-	-
	20	6.1	2.3	0.38	-	-
	21	4.8	7.1	1.5	-	-
Oxazole	**22**	0.48	13	27	-	-
	23	0.078	5.1	65	-	-
	24	0.62	>100	>161	-	-
Pyrazole	**25**	0.27	0.64	2.4	-	-
	26	2.3	2.2	0.96	52	23
ARI	**27**	0.58	9.5	16	-	-
	28	1.1	>20	>18	-	-
	29	2.3	12	5.2	-	-
Thiazole	**30**	0.18	0.92	5.1	-	-
	31	0.19	>100	>526	-	-
	32	0.088	3.0	34	-	-
AU1235	-	0.22	>100	454	6.0	27

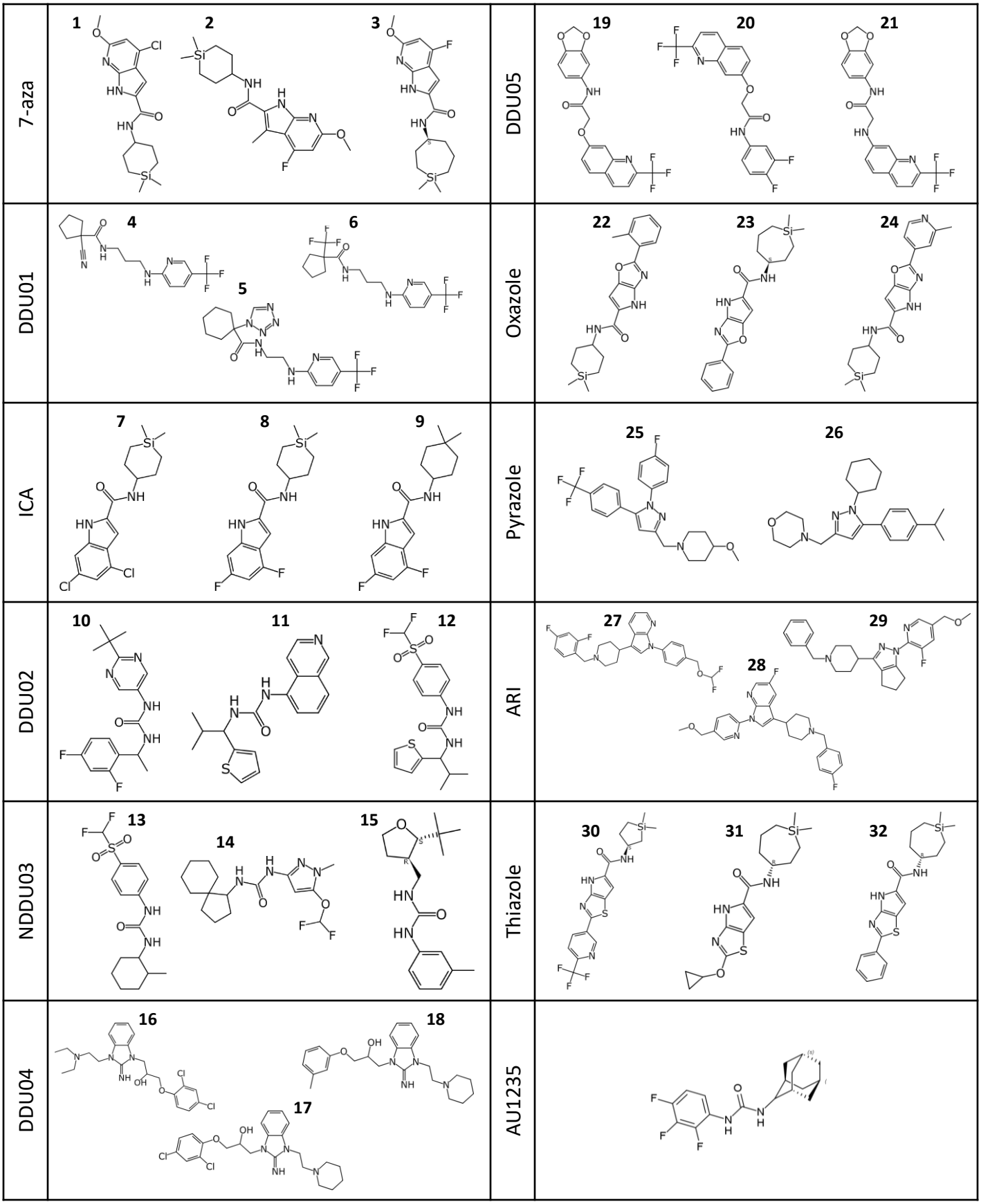


Figure 1: Chemical structures of MmpL3 inhibitors included in the study. Molecules are grouped by compound series.

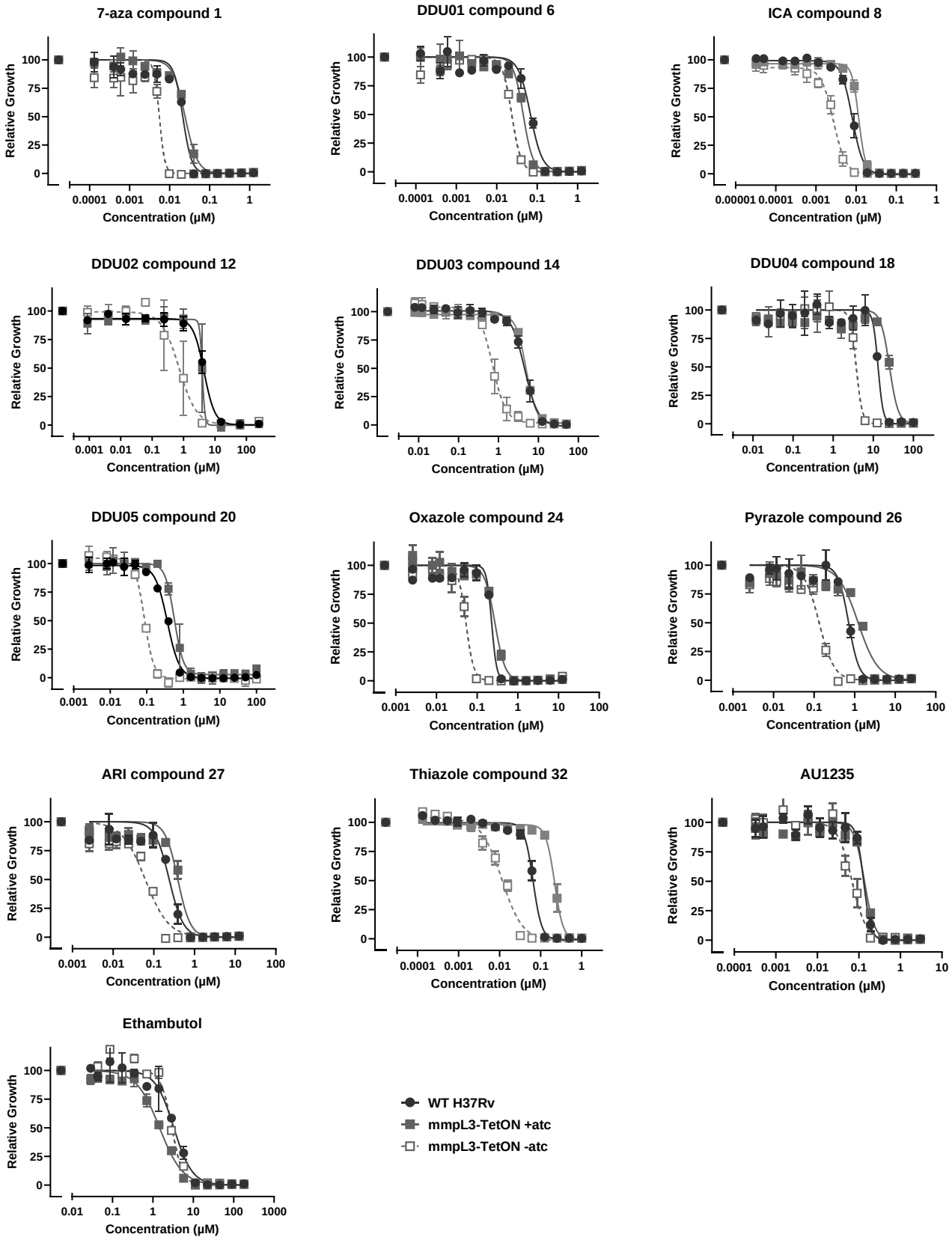


Figure 2: Differential expression of MmpL3 alters susceptibility to compounds. H37Rv (black circles) and the *mmpL3-TetON* strain with ATc (grey filled squares) or without ATc (grey empty squares) was exposed to compound for up to 14 days. OD_{580} was read. Plots shows mean and standard error for three independent experiments.

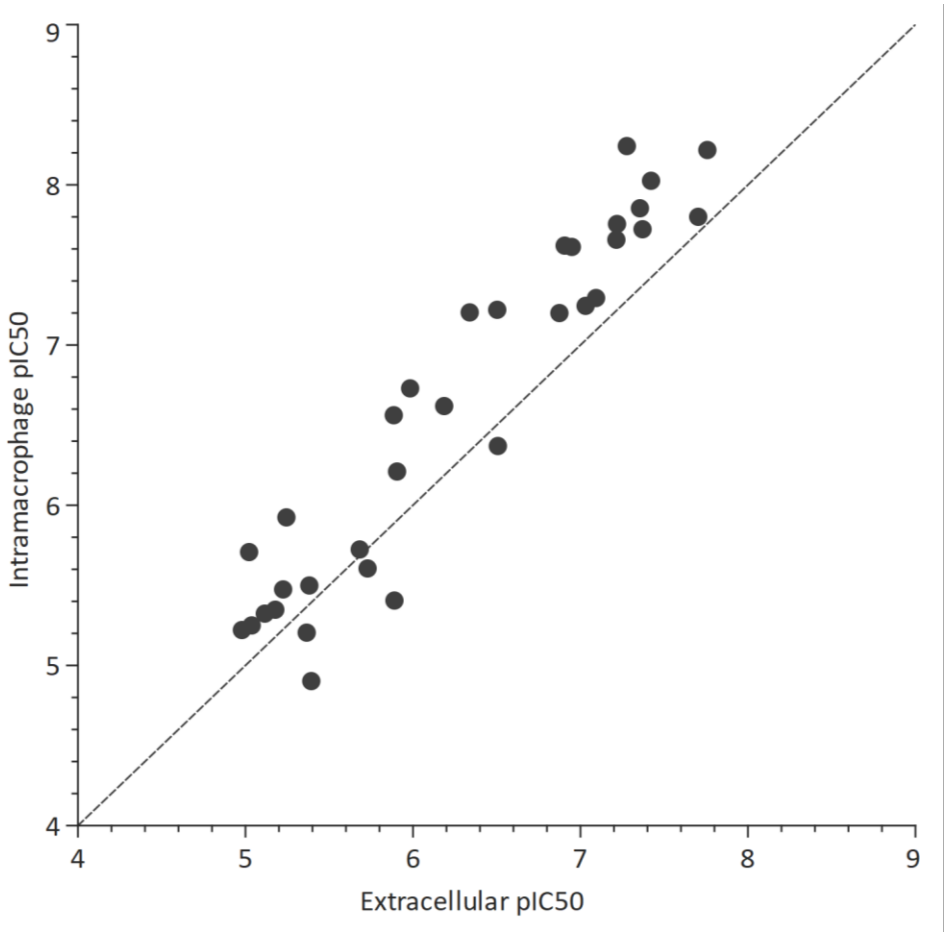


Figure 3: Activity of compounds against *M. tuberculosis* H37Rv-LP in axenic culture (extracellular) and infected THP-1 cells (intramacrophage). Points represent the geometric mean of at least two independent replicates. Dotted line shows $y = x$.

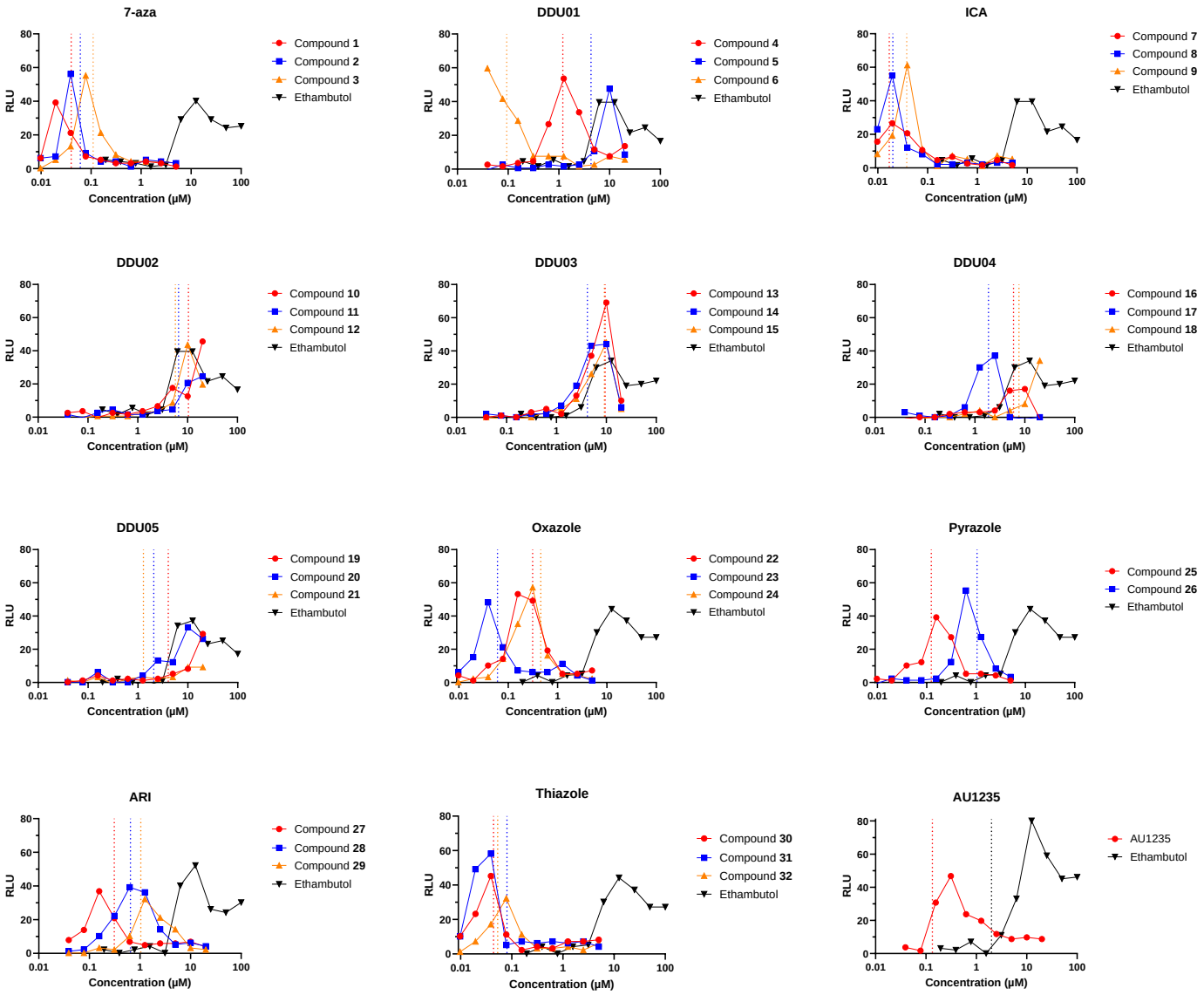


Figure 4: MmpL3 inhibitors induce cell wall stress. A *M. tuberculosis* strain expressing a luciferase operon under control of the cell wall stress-induced iniBAC promoter was exposed to compounds and luminescence (RLU) was measured. Ethambutol was used as a positive control. Plots show one of at least two independent experiments. Dotted vertical lines represent the IC_{50}.

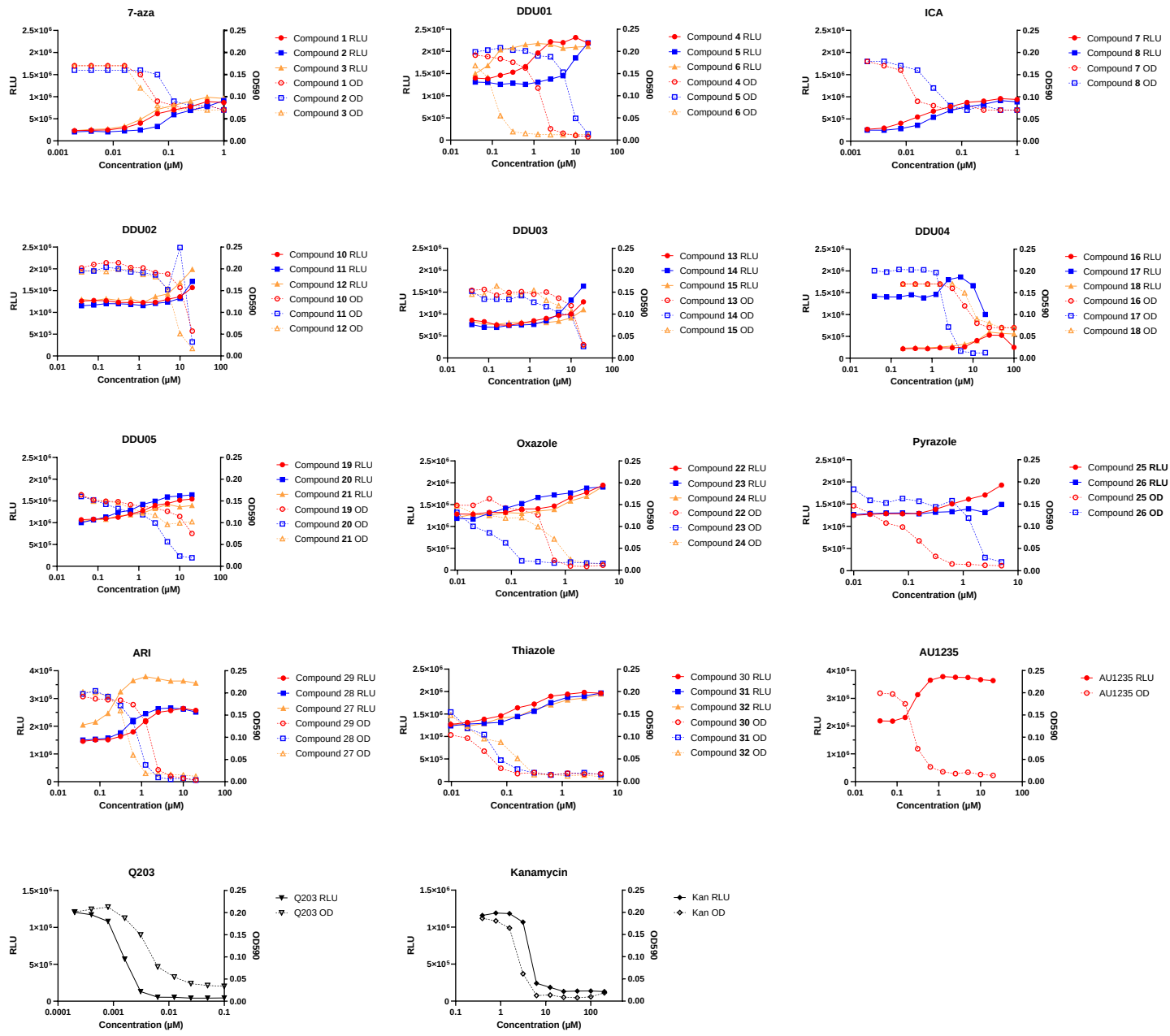
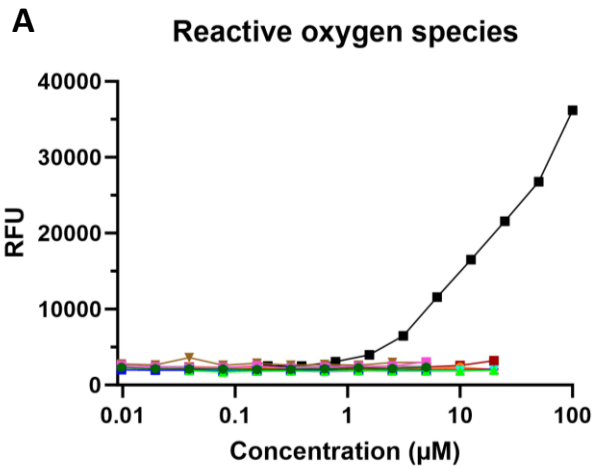
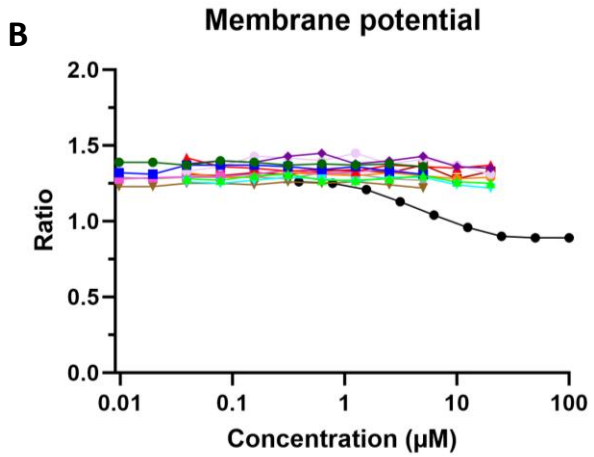
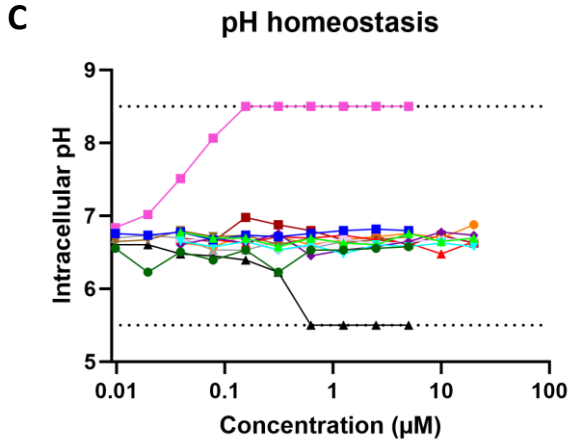


Figure 5: MmpL3 inhibitors induce an ATP boost. *M. tuberculosis* H37Rv-LP was exposed to compounds and ATP levels measured after 24 hours by addition of Bac-Titer Glo™ reagent and reading luminescence (RLU). OD_{590} was measured after 5 days. Q203 was used as a positive control (ATP depletion) and kanamycin was used as a negative control (no effect). Plot show one representative of at least two independent replicates.







Key	Compound	Series
	1	7-aza
	4	DDU01
	8	ICA
	12	DDU02
	14	DDU03
	18	DDU04
	20	DDU05
	24	Oxazole
	26	Pyrazole
	27	ARI
	32	Thiazole
	AU1235	-
	Econazole	-
	Monensin	-
	CCCP	-

Figure 6: MmpL3 inhibitors do not induce reactive oxygen species (ROS) production, disrupt membrane potential or maintenance of intracellular pH.

M. tuberculosis H37Rv-LP was exposed to compound. ROS was quantified using oxidation-sensitive fluorescent dye 2′,7′-Dichlorofluorescin diacetate (DCFDA) and measured fluorescence Ex485/Em535 nm (RFU).

Membrane potential was measured using cells stained with fluorescent probe 3,3'-Diethyloxacarbocyanine iodide (DiOC2(3)) and calculating the ratio between Ex488/Em530 nm and Ex488/Em530 nm fluorescence reads.

Intracellular pH was measured using an *M. tuberculosis* strain expressing a ratiometric GFP. Fluorescence was read at Ex390/Em505 nm and Ex475/Em510 nm and the ratio calculated. Intracellular pH was interpolated using a standard curve. Dotted lines represent the limit of intracellular pH measurement.

Plots show one representative of at least two independent experiments.