1 **Thienopyrimidine amide analogs target MmpL3 in *Mycobacterium tuberculosis***

2

3 Vanessa Pietrowski Baldin[a], Christopher L. Harding[a], Diana Quach[b], Joseph Sugie[b], Joe
4 Pogliano[b], Tanya Parish[a,c]

5

6 [a]Center for Global Infectious Disease Research, Seattle Children's Research Institute, Seattle,
7 United States.

8 [b]Linnaeus Bioscience, Inc., San Diego, USA.

9 [c]Department of Pediatrics, University of Washington School of Medicine, Seattle, USA.

Abstract

Objectives: The identification of novel agents with mechanisms of action distinct from those currently utilized in tuberculosis treatment remains a significant challenge. The mycobacterial protein MmpL3 has emerged as a promising drug target due to its essential role in the synthesis of the cell wall of *Mycobacterium tuberculosis*. We previously identified novel thienopyrimidine amides with good anti-tubercular activity.

Methods: We profiled a subset of thienopyrimidine amides determining activity against intracellular bacteria and bactericidal activity against replicating bacteria. We ran assays to determine mode of action by measuring cell wall stress, ATP production, and bacterial cytological profiling. We determined activity against a strain of M. tuberculosis with mutations in MmpL3. We isolated and sequenced resistant mutants.

Results: We tested five analogs against a strain of *M. tuberculosis* with mutations in MmpL3 and determined that they lost potency. Analogs induced P_{iniBAC}, a reporter for cell wall stress, and led to an ATP boost characteristic of cell wall inhibitors. Bacterial cytological profiling of a representative compound revealed a morphological profile consistent with other MmpL3 inhibitors.

Conclusions: Together, our data support MmpL3 as the most probable drug target for the TPA analogs and add to the growing list of scaffolds that can inhibit this vulnerable transporter.

Introduction

Despite the availability of drug treatment regimens, tuberculosis remains a major global health concern, with an estimated 8.2 million new cases reported in 2023, including 400,000 cases of drug resistant tuberculosis (1). Currently, no vaccine provides effective protection against pulmonary TB. Additionally, the bacteria can enter a latent phase, where they display no symptoms, or cause symptoms that mimic other pathologies (2). Together, these factors highlight the urgent need for intensified efforts in TB control and the development of novel therapies, particularly those targeting drug-resistant bacilli and aiming to shorten the duration of treatment.

Mycobacterium tuberculosis is the causative agent of TB in humans. *M. tuberculosis* has a thick cell wall, which is composed by a highly hydrophobic bilayer of mycolic acids linked to arabinogalactan in turn linked to peptidoglycan. This structure forms a unique barrier against drugs and the host immune defenses (3–5). Hence, cell wall biosynthesis represents an attractive target for the development of new inhibitors.

MmpL3 (Rv0206c) is an essential transporter which is highly conserved across the *Mycobacterium* genus (4, 6, 7) and is the only member of the MmpL family that is essential in *M. tuberculosis* (2, 4, 7). Its essentiality is attributed to its role in the translocation of trehalose monomycolate (TMM) to the periplasmic space, where it serves as a precursor for the synthesis of trehalose dimycolate (TDM), a critical component of the cell envelope (4, 8). Given the important role of MmpL3 in constructing the mycobacterial cell wall, it has emerged as a high-value, druggable target, and at the moment, one of the most studied for anti-TB drug development (6). There are numerous MmpL3 inhibitors with diverse chemical structures which have potent antibacterial activity (5, 9).

51 We previously identified and characterized novel thienopyrimidine amide (TPA) analogs that
52 inhibit *M. tuberculosis* growth and had low cytotoxicity. From this series, we identified two
53 subsets. One subset (TPA-L) had increased activity against a LepB (signal peptidase)
54 hypomorph, indicating that the signal peptidase or protein secretion is the target or mode of
55 action (10, 11). The second subset of molecules (TPA-M) had excellent potency but were
56 equipotent against wild-type and hypomorph strains of *M. tuberculosis* suggesting that the target
57 was not protein secretion. In this paper we explored the biological profile of the TPA-M series of
58 molecules and identified MmpL3 as the most likely intracellular target.

59 **Material and Methods**

60 **Bacterial strains and culture conditions**

61 *M. tuberculosis* H37Rv-LP ATCC 25618 (wild-type) (12) and *M. tuberculosis* LP-0497754-
62 RM301 (MmpL3 F255L, V646M, F644I) (13) were cultured in Middlebrook 7H9 broth medium
63 supplemented with 10% oleic acid, albumin, dextrose, and catalase (OADC) enrichment
64 (BBL/Beckton and Dickson) and 0.05% w/v Tween 80 (7H9-Tw-OADC). Nutrient-starved cells
65 were generated by resuspending cells in phosphate-buffered saline (PBS) with 0.05% w/v
66 Tyloxapol (PBS-Tyl) at OD 1.0 and incubating for 7 days at 37°C without agitation. Avirulent *M.*
67 *tuberculosis* $mc^2$6206 (Δ*pan*CD Δ*leu*CD) was grown in 7H9-Tw-OADC supplemented with 0.5%
68 wt/vol glycerol, 0.2% wt/vol Casamino Acids, 48 µg/mL pantothenate, and 50 µg/mL leucine at
69 30°C.

70 **Determination of minimum inhibitory concentration (MIC)**

71 TPA molecules (**Figure 1**) were synthesized as previously described (10). Compound stocks
72 were resuspended in DMSO and stored at -20°C. MICs were determined in 96- or 384-well
73 microplates as described (14). Briefly, *M. tuberculosis* was grown to mid-log phase; cultures

74 were dispensed into plates containing test compounds to a final OD_{590} of 0.02 and incubated at
75 37°C for 5 days (H37Rv) or 7 days ($mc^2$6206). The OD_{590} was measured and IC_{90} determined
76 as the concentration at which 90% of growth was inhibited as compared to controls (DMSO
77 only).

78 **Determination of activity against intracellular bacilli**

79 THP-1 cells were cultivated in RPMI-1640 medium supplemented with 10% FBS and incubated
80 at 37°C, 5% CO_2. Cells were treated with 80 nM PMA for 24 h prior to infection, harvested using
81 Accumax™ solution, and resuspended in fresh cRPMI at a final density of 9×10^5 cells/mL. Cells
82 were infected overnight at a multiplicity of 1:1 with *M. tuberculosis* expressing LuxABCDE and
83 exposed to compounds in 96-well plates for 72 h at 37°C and 5% CO_2. Bacterial viability was
84 assessed by luminescence; IC_{50} was determined as the concentration at which 50% of growth
85 was inhibited as compared to controls (DMSO only).

86 **Determination of bactericidal activity**

87 *M. tuberculosis* H37Rv-LP was cultured in 7H9-Tw-OADC or starved for 7 days in PBS-Tyl,
88 adjusted to a theoretical OD_{590} of 0.02 and exposed to compounds in 96-well plates. Cultures
89 were spotted onto 7H10 agar supplemented with v/v 10% OADC (7H10-OADC) on day 0, 7 and
90 14. The MBC was defined as the lowest concentration at which no visible growth was observed.
91 Niclosamide was included as a control.

92 **Induction of cell wall stress**

93 *M. tuberculosis* strain carrying the P_{iniBAC}-lux reporter plasmid (15) was grown to mid-log in
94 GAST/Fe protein-free medium containing 0.3 g/L Bacto Casitone, 0.05 g/L ferric ammonium
95 citrate, 4 g/L dibasic anhydrous potassium phosphate, 2 g/L citric acid, 1 g/L L-alanine, 1.2 g/L
96 magnesium chloride, 0.6 g/L potassium sulfate, 2 g/L ammonium chloride, 1.8 mL of 10 M

97 sodium hydroxide, 10 mL glycerol, 5 mL 10% Tween 80, plus 15 µg/mL kanamycin. Cultures
98 were adjusted to a theoretical OD_{590} of 0.02 and used to inoculate 96-well plates containing
99 compounds. After 72 hours of incubation at 37°C, 1 vol of 10 mg/mL luciferin in of 1M HEPES
100 buffer pH 7.8, 1M DTT was added and RLU was read after 25 min of incubation at RT.
101 Ethambutol was included as a positive control.

102 **Determination of ATP levels**

103 ATP was measured using the BacTiter-Glo Assay kit (Promega) according to the manufacturer's
104 instructions. Log phase *M. tuberculosis* was exposed to compounds for 24 h, .50 µL BacTiter-
105 Glo™ was added, incubated at RT for 10 min and RLU read. Growth was measured after 120 h
106 by OD. Q203, was included as a positive control.

107 **Isolation of resistant mutants**

108 Log phase *M. tuberculosis* was plated on 7H10-OADC plates with 5X solid MIC and 10X solid
109 MIC. Plates were incubated at 37°C until isolated colonies appeared. Potential resistant mutants
110 were picked and streaked onto 5X MIC. MIC in liquid medium was measured to confirm
111 resistance. Genomic DNA from three isolates was extracted from cultured cells by heat
112 inactivation for 10 min at 100°C followed by 0.22 µm filtration. PCR amplification was performed
113 using 10 µL of extract DNA in a final volume of 50 µL containing 1 µL of Pfu polymerase
114 (Agilent), 5 µL of 10X Pfu amplification buffer (Agilent), 2.5 µL of primers MmpL3_seq_1_fwd
115 (5'-gattcgctacctgagcag-3') and MmpL3_seq_11_rev (0.5 µM) (5'-catttactgcagccgctg-3') and 4 µL
116 of 10 mM dNTP mix. PCR amplification was conducted, products were purified using the Qiagen
117 PCR purification kit, and sequencing was performed by Plasmidsaurus using Oxford Nanopore
118 Technology with custom analysis and annotation.

119 **Bacterial Cytological Profiling (BCP)**

120 *M. tuberculosis* mc^{2}6206 for bacterial cytological profiling was prepared as described (16).
121 Briefly, cultures were adjusted to an OD_{600} of ~0.06-0.08 and incubated at 30°C for 18-20 hours
122 before exposure to compounds at 1X and 5X MIC for 48h and 120 hours. Cells were fixed using
123 a mixture of 100 µL of 16% paraformaldehyde, 3 µL of 8% glutaraldehyde, and 20 µL of 0.4 M
124 phosphate buffer pH 7.5, washed twice with 200 µL of warm medium, concentrated to
125 approximately 30 µL, and stained for 30 min. Full-field fluorescence microscopy images of the
126 samples were preprocessed from their original proprietary microscope file format into a common
127 image format (TIFF). The original image dimensions (3×2048×2048) were cropped by trimming
128 124 pixels from each edge to avoid optical artifacts, resulting in dimensions of 3×1800×1800. A
129 600-pixel square sliding window was passed over this image with a step size of 60 pixels to
130 produce 400 sub-images, which were each passed to a trained convolutional neural network
131 (CNN). Each full-field image, consisting of 400 sub-images, generated a vector that describes a
132 point in latent space. Similarity scores were calculated by comparing the location of unknown
133 compound-treated cells in this space to control compound-treated cells using a scaled version
134 of the average minimum distance.

135 **Results and Discussion**

136 **TPA analogs are active against intracellular mycobacteria**

137 We previously demonstrated that the TPA series of compounds are active against aerobically-
138 grown *M. tuberculosis* (10). A subset of analogs had potent anti-tubercular activity. Since these
139 analogs had equipotent activity against wild-type and LepB hypomorph strains, we hypothesized
140 that they did not target protein secretion. We wanted to determine the mode of action and target
141 of these potent molecules.

142 We had already noted that compounds were active against replicating bacteria, so we expanded
143 our work to look at activity against intracellular bacteria and non-replicating bacteria. We
144 selected several active analogs (Figure 1). Molecules were tested for activity against
145 intracellular *M. tuberculosis* in THP-1 cells. All compounds were active against intracellular *M.*
146 *tuberculosis*, with activity similar to the MICs (Table 1).

147

148 **TPA analogs are bactericidal against replicating bacilli**

149 We assessed the bactericidal activity of the TPA analogs against *M. tuberculosis* wild type
150 following a 14-day exposure under replicating conditions as well as against non-replicating
151 bacteria generated by nutrient starvation. All analogs were bactericidal against replicating *M.*
152 *tuberculosis* with MBC/MIC ratio of <4. No bactericidal activity was seen against non-replicating
153 cells (MBC > 100 μM) (**Table 1**). These data are in contrast to what we previously noted with
154 inhibitors of LepB which showed bactericidal activity against non-replicating bacteria further
155 supporting a different target or mode of action (17).

156 **TPA analogs induce cell wall stress in *M. tuberculosis***

157 We were interested in determining the mode of action of our molecules. As a first step, we
158 determined whether molecules induced cell wall stress. We used a reporter strain of M.
159 tuberculosis expressing luciferase under the control of P_{iniBAC}, since Induction of *iniBAC*
160 expression is a marker of cell envelope stress (18). We saw induction of PiniBAC for all
161 analogs, which predominantly occurred at concentrations close to the MIC. These data suggest
162 that TPA analogs cause cell wall stress (Figure 2 and Figure S1).

163 **TPA analogs boost ATP in M. tuberculosis**

164 A link between induction of *iniBAC* and a burst in ATP production has previously been proposed
165 (19). To determine if our molecules also affect ATP levels, we measured intracellular ATP levels
166 following treatment with TPA analogs. As anticipated, increasing compound concentrations led
167 to a boost in ATP levels (Figure 3 and Figure S2). Again we saw the boost occurring at
168 concentrations around the MIC. These data are consistent with the response seen with other
169 MmpL3 inhibitors (3, 20).

170 **MmpL3 mutation leads to resistance to TPA analogs**

171 We hypothesized that our molecules could be targeting MmpL3, since this is a highly
172 promiscuous target and cell wall stress and elevated intracellular ATP levels are features
173 associated with MmpL3 inhibition in *M. tuberculosis* (3). We compared TPA activity against the
174 wild-type *M. tuberculosis* and strain with mutations in MmpL3 which is resistant to a wide range
175 of MmpL3 inhibitors (13, 20). The MmpL3 mutant strain demonstrated increased resistance to
176 all analogs, with at least a 4-fold increase in IC_{90} (Table 1). Interestingly, this strain was highly
177 resistant to three of the five analogs which lost all activity (IC_{90} >50-100 μM). Thus our data are
178 consistent with MmpL3 being the drug target for this compound series.

179 **Resistant strains have a non-synonymous mutation in *mmpL3***

180 In order to determine whether there might be additional targets, we isolated resistant mutants
181 using TPN-0089300. Three strains were selected with an IC_{90} of >100 μM. Since MmpL3
182 mutations lead to resistance, we sequenced *mmpL3* in three resistant isolates (RM3, RM4, and
183 RM6). All isolates had the same non-synonymous mutation in MmpL3 of F644L. This mutation
184 has been associated with resistance to other MmpL3 inhibitors and is similar to one of the
185 mutations in the MmpL3 mutant strain we used to test for resistance (F644I) (4, 8, 13, 21).

186 **Cytological profiling**

187 We determined the phenotypic effect of a representative TPA analog using bacterial cytological
188 profiling (16). We confirmed that TPN-0089300 was active against the avirulent *M. tuberculosis*
189 strain $mc^2$6206 (MIC of 1 µg/mL). Bacterial cells were exposed to TPN-0089300 for 48 and 120
190 hours at 2X and 5X MIC (2 µg/mL and 5 µg/mL respectively). Morphological profiling
191 demonstrated that bacterial morphology changed with cells becoming more rounded and losing
192 membrane integrity after 120h (Figure 4). Based on the similarity score, this profile was
193 identified as a strong match to other MmpL3 inhibitors (Figure 5) (16, 20).

194 **Conclusions**

195 We provide strong evidence that a subset of TPA analogs, which we now term TPA-M, target
196 MmpL3 in *M. tuberculosis*. TPA-M compounds were bactericidal for replicating bacilli, induced
197 cell walls stress and boosted ATP production. Mutations in MmpL3 conferred resistance and
198 bacterial cytological profiling identified MmpL3 as the most likely mode of action. We
199 demonstrated that the TPA-M analogs do not kill starved, non-replicating bacilli. This is
200 consistent with other MmpL3 inhibitors that have little effect against non-replicating *M.*
201 *tuberculosis* (3, 22). Cells treated with MmpL3 inhibitors develop dimples at the dividing pole,
202 where the protein localizes during active cell division and the lack of activity against non-
203 replicating cells has been attributed to the absence of MmpL3 localization at the pole in the non-
204 replicating state (7, 23). Taken together our data suggest that MmpL3 is the primary target of the
205 TPA-M series.

206 **Acknowledgments**

207 The authors kindly thank Grace Liu, Renee Allen, and Lauren Ames for technical assistance.

Funding Sources

This work was supported by the Department of Defense office of the Congressionally Directed Medical Research Programs under award number under award number PR180794 and by NIAID of the National Institutes of Health under award number R01AI182006. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest

The authors declare no competing interests.

References

1. World Health Organization. 2024. Global tuberculosis report 2024. https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2024

2. Diab A, Dickerson H, Al Musaimi O. 2025. Targeting the Heart of Mycobacterium: Advances in Anti-Tubercular Agents Disrupting Cell Wall Biosynthesis. Pharmaceuticals 18:70.

3. Ames L, Allen R, Boshoff HI, Cleghorn L, Engelhart C, Schnappinger D, Parish T. Common biological features of Mycobacterium tuberculosis MmpL3 inhibitors.

4. Kapp E, Calitz H, Streicher EM, Dippenaar A, Egieyeh S, Jordaan A, Warner DF, Joubert J, Malan SF, Sampson SL. 2023. Discovery and biological evaluation of an adamantyl-amide derivative with likely MmpL3 inhibitory activity. Tuberculosis 141:102350.

5. North EJ, Schwartz CP, Zgurskaya HI, Jackson M. 2023. Recent advances in mycobacterial membrane protein large 3 inhibitor drug design for mycobacterial infections. Expert Opin Drug Discov 18:707–724.

6. Adams O, Deme JC, Parker JL, Fowler PW, Lea SM, Newstead S. 2021. Cryo-EM structure and resistance landscape of M. tuberculosis MmpL3: An emergent therapeutic target. Structure 29:1182-1191.e4.

7. Williams JT, Abramovitch RB. 2023. Molecular Mechanisms of MmpL3 Function and Inhibition. Microb Drug Resist 29:190–212.

8. Williams JT, Haiderer ER, Coulson GB, Conner KN, Ellsworth E, Chen C, Alvarez-Cabrera N, Li W, Jackson M, Dick T, Abramovitch RB. Identification of New MmpL3 Inhibitors by Untargeted and Targeted Mutant Screens Defines MmpL3 Domains with Differential Resistance.

9. Chauhan M, Barot R, Yadav R, Joshi K, Mirza S, Chikhale R, Srivastava VK, Yadav MR, Murumkar PR. 2024. The *Mycobacterium tuberculosis* Cell Wall: An Alluring Drug Target for Developing Newer Anti- TB Drugs—A Perspective. Chem Biol Drug Des 104:e14612.

10. Lynde BE, Chemaly DM, Baldin VP, Greve E, Harding CL, Graner JM, Hardy M, Chowdhury S, Parish T. 2025. Novel 3-Aminothieno[2,3- *b*]pyridine-2-carboxamides with Activity against *Mycobacterium tuberculosis*. ACS Med Chem Lett 16:241–249.

11. Bonnett SA, Ollinger J, Chandrasekera S, Florio S, O'Malley T, Files M, Jee J-A, Ahn J, Casey A, Ovechkina Y, Roberts D, Korkegian A, Parish T. 2016. A Target-Based Whole Cell Screen Approach To Identify Potential Inhibitors of *Mycobacterium tuberculosis* Signal Peptidase. ACS Infect Dis 2:893–902.

12. Ioerger TR, Feng Y, Ganesula K, Chen X, Dobos KM, Fortune S, Jacobs WR, Mizrahi V, Parish T, Rubin E, Sassetti C, Sacchettini JC. 2010. Variation among genome sequences of H37Rv strains of Mycobacterium tuberculosis from multiple laboratories. J Bacteriol 192:3645–3653.

13. McNeil MB, O'Malley T, Dennison D, Shelton CD, Sunde B, Parish T. 2020. Multiple Mutations in Mycobacterium tuberculosis MmpL3 Increase Resistance to MmpL3 Inhibitors. mSphere 5:e00985-20.

14. Ollinger J, Bailey MA, Moraski GC, Casey A, Florio S, Alling T, Miller MJ, Parish T. 2013. A Dual Read-Out Assay to Evaluate the Potency of Compounds Active against Mycobacterium tuberculosis. PLoS ONE 8:e60531.

15. Naran K, Moosa A, Barry CE, Boshoff HIM, Mizrahi V, Warner DF. 2016. Bioluminescent Reporters for Rapid Mechanism of Action Assessment in Tuberculosis Drug Discovery. Antimicrob Agents Chemother 60:6748–6757.

16. Quach D, Sharp M, Ahmed S, Ames L, Bhagwat A, Deshpande A, Parish T, Pogliano J, Sugie J. 2025. Deep learning–driven bacterial cytological profiling to determine antimicrobial mechanisms in *Mycobacterium tuberculosis*. Proc Natl Acad Sci 122:e2419813122.

17. Bonnett SA, Dennison D, Files M, Bajpai A, Parish T. 2018. A class of hydrazones are active against non-replicating Mycobacterium tuberculosis. PloS One 13:e0198059.

18. Alland D, Steyn AJ, Weisbrod T, Aldrich K, Jacobs WR. 2000. Characterization of the *Mycobacterium tuberculosis iniBAC* Promoter, a Promoter That Responds to Cell Wall Biosynthesis Inhibition. J Bacteriol 182:1802–1811.

19. Shetty A, Dick T. 2018. Mycobacterial Cell Wall Synthesis Inhibitors Cause Lethal ATP Burst. Front Microbiol 9:1898.

20. Allen R, Ames L, Baldin VP, Butts A, Henry KJ, Durst G, Quach D, Sugie J, Pogliano J, Parish T. 2024. An arylsulfonamide that targets cell wall biosynthesis in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 68:e01037-24.

21. Ioerger TR, O'Malley T, Liao R, Guinn KM, Hickey MJ, Mohaideen N, Murphy KC, Boshoff HIM, Mizrahi V, Rubin EJ, Sassetti CM, Barry CE, Sherman DR, Parish T, Sacchettini JC. 2013. Identification of New Drug Targets and Resistance Mechanisms in Mycobacterium tuberculosis. PLoS ONE 8:e75245.

22. Rao SPS, Lakshminarayana SB, Kondreddi RR, Herve M, Camacho LR, Bifani P, Kalapala SK, Jiricek J, Ma NL, Tan BH, Ng SH, Nanjundappa M, Ravindran S, Seah PG, Thayalan P, Lim SH, Lee BH, Goh A, Barnes WS, Chen Z, Gagaring K, Chatterjee AK, Pethe K, Kuhen K, Walker J, Feng G, Babu S, Zhang L, Blasco F, Beer D, Weaver M, Dartois V, Glynne R, Dick T, Smith PW, Diagana TT, Manjunatha UH. 2013. Indolcarboxamide Is a Preclinical Candidate for Treating Multidrug-Resistant Tuberculosis. Sci Transl Med 5.

23. Lun S, Tasneen R, Chaira T, Stec J, Onajole OK, Yang TJ, Cooper CB, Mdluli K, Converse PJ, Nuermberger EL, Raj VS, Kozikowski A, Bishai WR. 2019. Advancing the Therapeutic Potential of Indoleamides for Tuberculosis. Antimicrob Agents Chemother 63:e00343-19.

289

290

	IC_{90} (µM)	IC_{90} (µM)			MBC (µM)	
Molecule ID	**Intracellular**	**Wild-type**	**RM301**	**Fold-change**	**Replicating**	**Non-replicating**
TPN-0102024	1.5 ± 0.21	1.5 ± 1.0	>100	>70	4.1 ± 1.8	>100
TPN-0099994	0.26 ± 0.03	0.7 ± 0.19	3.5 ± 2.4	6	2.3 ± 1.3	>100
TPN-0099934	2.7 ± 0.21	7.5 ± 3.3	>50	7	21 ± 7.2	>100
TPN-0099730	1.3 ± 0.47	1.2 ± 0.5	>100	>90	3.6 ± 2.4	>100
TPN-0089300	0.57 ± 0.33	1.2 ± 0.6	4.2 ± 0.6	4	3.1 ± 0	>100

291
292

293

294 **Table 1. Activity of TPA analogs against *M. tuberculosis*.**

295 Intracellular IC_{90} = concentration that leads to 90% inhibition of bacterial replication in THP-1
296 macrophages. Data are the average and standard deviation of two independent replicates. IC_{90}
297 = concentration that leads to 90% inhibition of bacterial growth in aerobic culture. Data are the
298 average and standard deviation of a minimum of 3 replicates. Fold-change = comparison of IC_{90}
299 for wild-type and RM301 (MmpL3 F255L, V646M, F644I) strains. MBC = minimum bactericidal
300 concentration – concentration at which no viable bacteria were detected after 14 days. Data are
301 the average and standard deviation of three replicates.

302

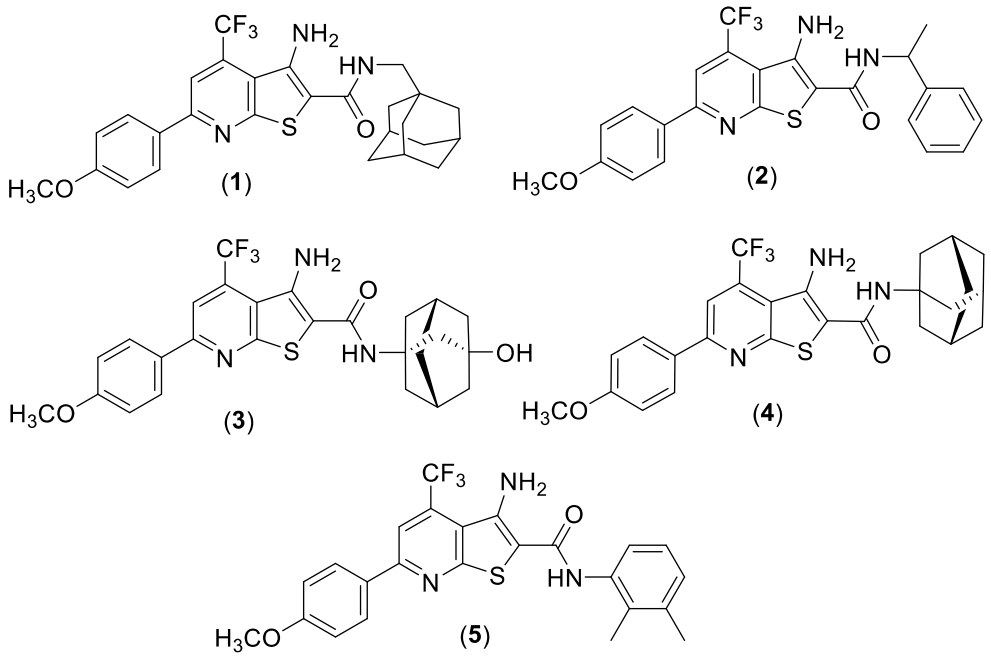


Figure 1. Structure of analogs used in this study. (1) TPN-0102024 (2) TPN-0099994 (3) TPN-0099934 (4) TPN-0099730 (5) TPN-0089300

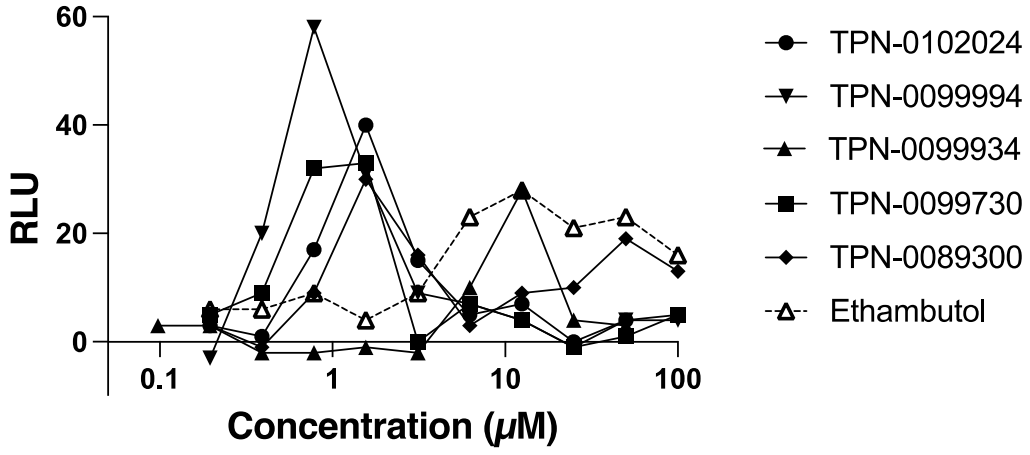


Figure 2. Exposure to TPA analogs induces cell wall stress in *M. tuberculosis*.

M. tuberculosis P_{iniBAC}-Lux was exposed to compounds for 72h and luminescence was read. Data are representative of two independent experiments (see Fig S1).

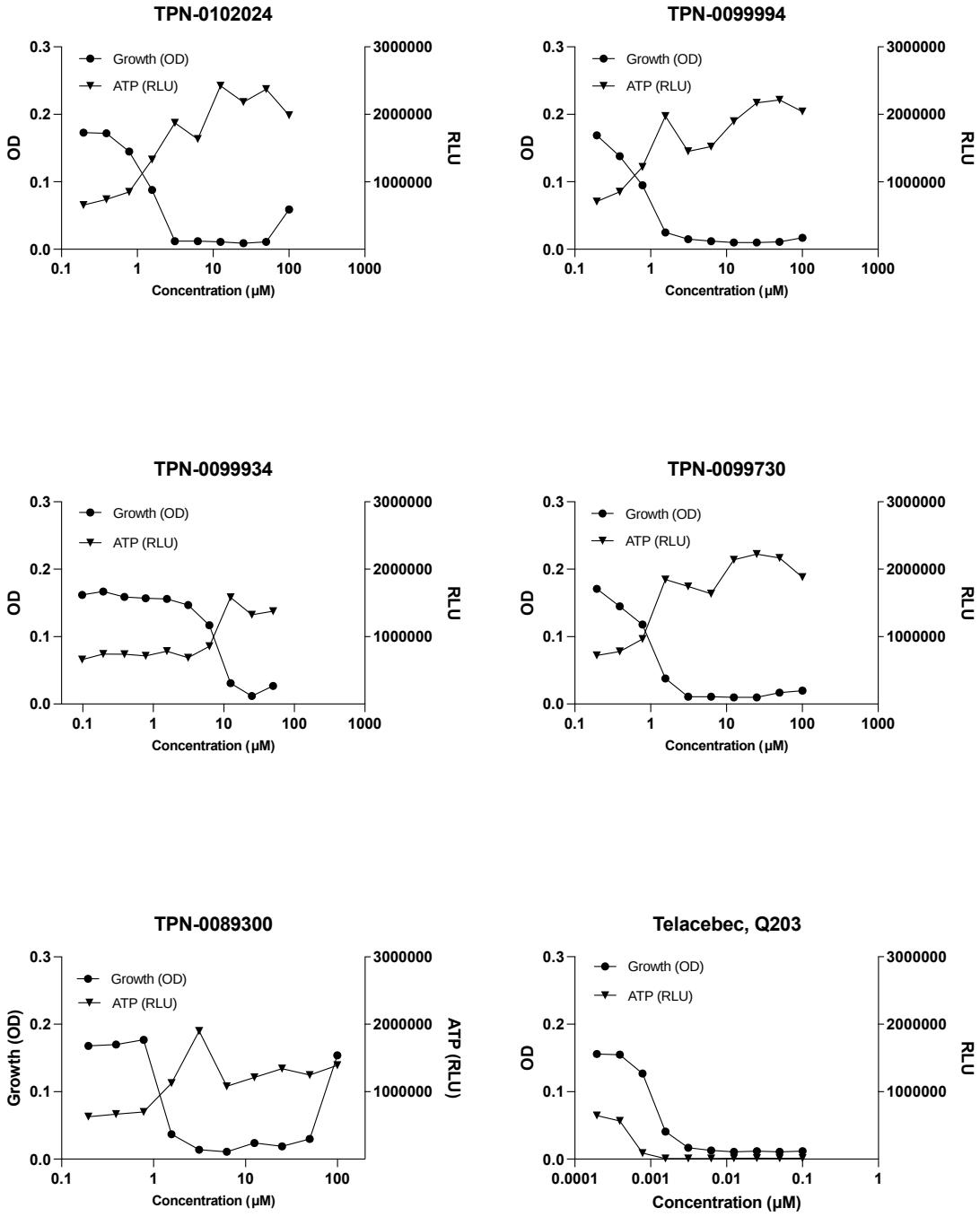


Figure 3. TPA analogs boost ATP in *M. tuberculosis*.

M. tuberculosis was exposed to compounds for 24h and ATP was measured using BacTiter-Glo. Growth was measured by OD after 5 d. Data are representative of two independent experiments (see Fig S2). Q203 was used as a control.

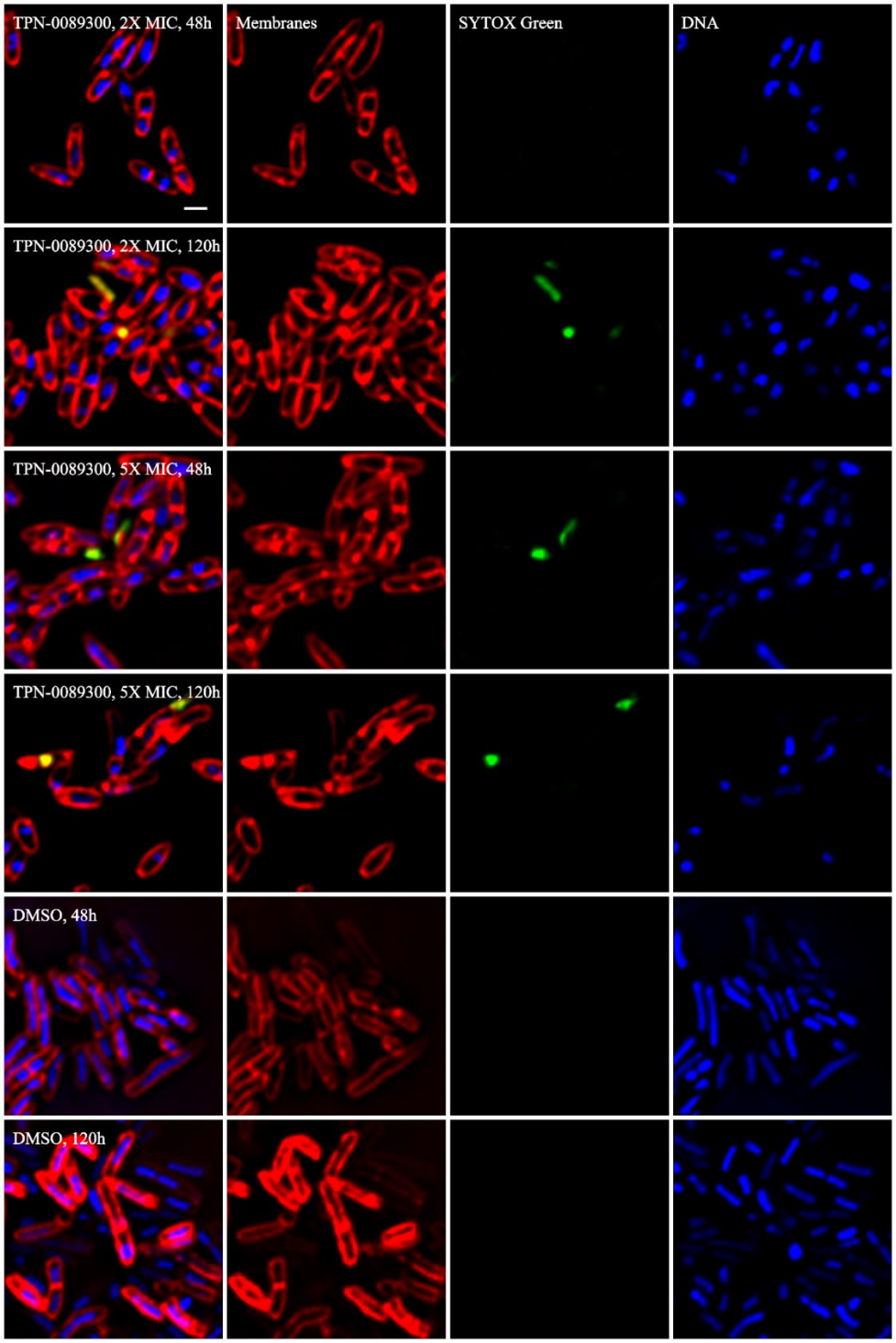
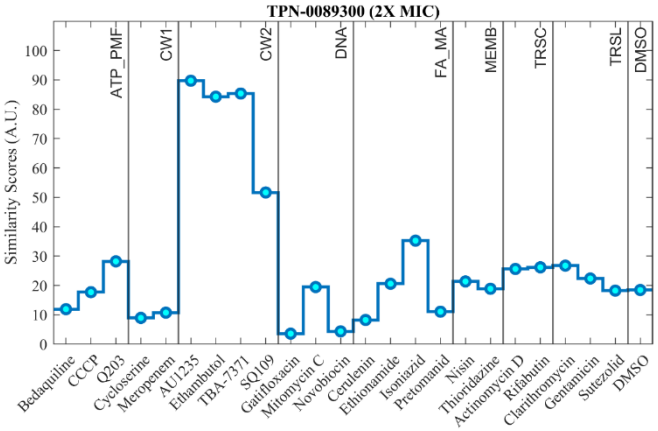
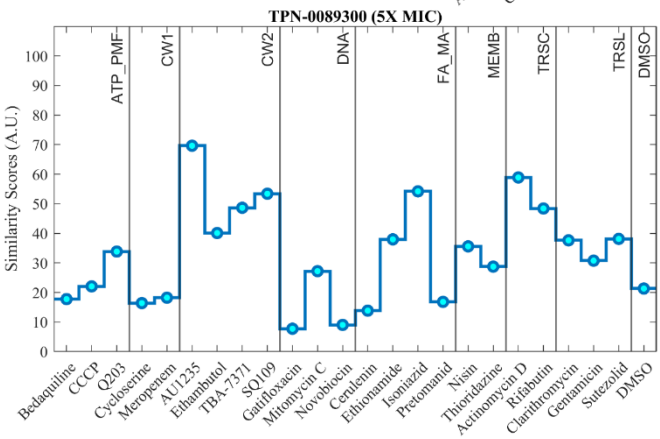


Figure 4. Cytological profiling in response to TPN-0089300. *M. tuberculosis* mc^{2}6206 was treated for 48 and 120 h with TPN-0089300 or DMSO (control) and stained. Membranes are shown in red, DNA in blue and green indicates loss of membrane integrity.





	ATP_PMF			CW1		CW2				DNA			FA_MA				MEMB		TRSC		TRSL			DMSO
	Bedaquiline	CCCP	Q203	Cycloserine	Meropenem	AU1235	Ethambutol	TBA-7371	SQ109	Gatifloxacin	Mitomycin_C	Novobiocin	Cerulenin	Ethionamide	Isoniazid	Pretomanid	Nisin	Thioridazine	Actinomycin_D	Rifabutin	Clarithromycin	Gentamicin	Sutezolid	DMSO
TPN-0089300 (2X MIC)	12	18	28	9	11	90	84	85	52	4	19	4	8	21	35	11	21	19	26	26	27	22	18	18
TPN-0089300 (5X MIC)	18	22	34	16	18	70	40	49	53	8	27	9	14	38	54	17	36	29	59	48	38	31	38	21

328 **Figure 5. Similarity scores for cytological profiling in response to TPN-0089300.**