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Three-dimensional culture modelling reveals divergent *Mycobacterium* *tuberculosis* virulence and antimicrobial treatment response
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Abstract

Tuberculosis (TB) remains a persistent epidemic, and the emergence of drug-resistant *Mycobacterium tuberculosis* (Mtb) presents a global healthcare threat. Whilst some new agents have been successfully introduced, innovative technologies to evaluate emerging anti-TB compounds are required to inform transformative approaches. Mtb is an obligate human pathogen, and consequently utilizing models that are consistent with human disease is likely to be critical. We have developed a human 3-dimensional (3-D) cell culture model that reflects human disease gene expression patterns and causes Mtb to become pyrazinamide sensitive *in vitro*. Here, we identify key differences in virulence between the standard laboratory strain, Mtb H37Rv, and clinical isolates. We demonstrate that Mtb H37Rv is attenuated in the 3-D system, more susceptible to antibiotics and hyper-inflammatory compared to clinical isolates. Prolonged *in vitro* culture of a clinical strain leads to attenuation. We then characterise antibiotic sensitivity of multi-drug-resistant Mtb within the 3-D model and define relative bactericidal activity. Finally, we demonstrate that verapamil increases efficacy of bedaquiline and delamanid antibiotic therapy. Taken together, our findings suggest that studying virulent clinical strains in an advanced cell culture system is a powerful adjunct to established methodologies to evaluate new interventions for TB.

45 # Introduction

46 Tuberculosis (TB) continues to be a global epidemic, killing approximately 1.5 million
47 people annually, and unfortunately the COVID-19 pandemic is likely to significantly worsen
48 TB control (1). Furthermore, *Mycobacterium tuberculosis* (Mtb), the causative bacterium, is
49 becoming progressively more resistant to antibiotics (2). Standard treatment takes a
50 minimum of 6 months, and MDR-TB patients often require longer treatment for 18 months,
51 with extensive drug side effects (3). Consequently, there is a pressing need for shortening
52 therapy and discovering new chemotherapeutics (4).

53 Mtb H37Rv is a standard laboratory reference strain, which is very widely used for
54 experimental purposes around the world. However, it was last isolated from a patient in 1905
55 (5, 6) and has been repeatedly sub-cultured in broth culture since then. Consequently, H37Rv
56 has been widely proposed to be attenuated compared to clinical strains (7, 8). Modern
57 circulating Mtb strains have been classified into multiple lineages (9), and some, such as
58 Lineage 2 Beijing isolates, have contributed to large outbreaks of TB globally (10, 11).

59 Standard TB treatment involves four antibiotics initially, rifampicin, isoniazid, pyrazinamide
60 and ethambutol for two months, followed by rifampicin and isoniazid for four months.
61 Treatment of drug resistant Mtb requires longer courses of antibiotics, typically for greater
62 than 12 months, with second-line agents often having lesser killing efficacy or a worse side-
63 effect profile, such as moxifloxacin, D-cycloserine, para-amino salicylic acid, linezolid and
64 amikacin. The most recently approved agents are bedaquiline and delamanid (3, 12). Recent
65 studies of shorter treatments have had variable results (13-15), demonstrating that innovative
66 technologies and new methods to investigate mechanisms of novel antibiotics are needed to
67 predict optimal combinations of antimicrobial agents.

68 Enhanced drug resistance can be due to upregulation of bacterial efflux pumps, either through
69 mutation or increased activity (16-20). In the zebrafish model of *M. marinum* infection,
70 multi-drug tolerance develops in replicating mycobacteria by a macrophage-induced efflux
71 mechanism (21). Importantly, intra-macrophage residence of the bacteria contributed to the
72 phenomenon. Efflux pump inhibitors such as verapamil reduced tolerance to antibiotics
73 including isoniazid and rifampicin (22) and MDR strains developed macrophage-induced
74 drug tolerance and utilized efflux pumps for intracellular growth. Consequently, the calcium-
75 channel blocker verapamil has emerged as a potential adjunctive chemotherapy for TB (23-
76 25).

77 In this study, we investigate pathogenicity and antibiotic sensitivity of Mtb H37Rv compared
78 to clinical Mtb isolates in broth culture and a 3-D primary human cellular granuloma model
79 of TB infection, where host gene expression reflects events in patients more closely than
80 standard cell culture (26-28), and Mtb is pyrazinamide sensitive (29). We demonstrate
81 significant differences between laboratory and clinical strains in the 3-D system. We then
82 investigate MDR-TB, and emerging therapies and combination treatment, further
83 demonstrating the potential of adjunctive verapamil to increase antibiotic efficacy against
84 clinical isolates when tested in an advanced cell culture model.

85

86 Experimental procedures

87 Mycobacteria culturing and reagents

88 The reference strain *Mycobacterium tuberculosis* H37Rv originated from Prof. Friedland's
89 laboratory (Imperial College London), originally from the Pasteur institute, and Erdman and
90 CDC 1551 strains were a gift from Dr Rawkins at Public Health England, Porton Down, UK.
91 We obtained drug-sensitive clinical isolates and MDR-TB from Prof. Drobniewski (Imperial

92 College London) (11). Bioluminescent bacteria were routinely cultured in Middlebrook 7H9
93 medium (BD Biosciences, Oxford) supplemented with 10% ADC enrichment (SLS), 0.2%
94 glycerol and 0.02% Tween 80 with kanamycin (25µg/ml) at 37°C in an incubator with
95 shaking at 200 rpm. The 2-year old culture of the Beijing strain, 0414B lux, was generated
96 by continuously repeating weekly 200µl sub-culture into a freshly prepared 2ml broth for this
97 period. Bacteria were cultured until optical density of 0.6 was reached, which is equivalent to
98 about 1x 10^8 CFU/ml. Either spectrophotometer (WPA Biowave CO8000 cell density meter,
99 UK) or luminometer (GloMax® 20/20 Single Tube Luminometer; Promega, UK; detecting
100 luminescence) was used to observe mycobacterial proliferation in 7H9 media. Reagents in
101 this study were mainly Sigma-Aldrich acquired apart from delamanid and bedaquiline, which
102 were bought from Adooq Bioscience, and tariquidar, diltiazem, amlodipine and carbonyl
103 cyanide m-chlorophenyl hydrazine (CCCP), which were obtained from Tocris.

104

105 **Generating bio-luminescent mycobacteria by electroporation**

106 Bio-luminescent mycobacteria were generated by electroporation of pMV306hsp+LuxG13
107 plasmid (Addgene # 26161), into *M. tuberculosis* H37Rv, Erdman, CDC1551, clinical
108 isolates and MDR strains (Table S1) as previously described (30, 31). We used 7H11 agar
109 plates with kanamycin (25µg/ml) to select the strains transformed with an integrating vector.
110 Luminescence of five randomly picked colonies was recorded and the transformants were
111 checked by PCR with oligonucleotides (Forward: 5'- AACCGTATTACCGCCTTTGA-3'
112 and Reverse: 5'- TATCAGCCCGTACCAGCATT-3') amplifying the corresponding
113 promoter and reporter gene.

114

115

116 **Infection of human peripheral blood mononuclear cells**

117 Ethical approval was provided by the National Research Ethics Service Committee South
118 Central - Southampton A, ref. 13/SC/0043. The National Health Service Blood and
119 Transplant, Southampton, UK supplied us with single-donor buffy coats, from which
120 peripheral blood mononuclear cells (PBMCs) were isolated using density gradient
121 centrifugation over Ficoll-Paque (GE Healthcare Life Sciences, UK). Host cells were then
122 infected with luminescent mycobacteria at a multiplicity of infection (MOI) of 0.1. After
123 overnight incubation at 37°C in 5% CO_2, the infected PBMCs were treated with Versene
124 solution for 10 min and neutralised by HBSS without Ca/Mg (Gibco). The cells were
125 detached by scraping, placed in 50ml falcon tubes, topped up with HBSS and spun at 320xg
126 for 8 min at 4°C. The supernatant was then decanted and the remaining pellet was re-
127 suspended in RPMI 1640 solution with added 10μg/ml of ampicillin, 2mM of glutamine,
128 25μg/ml of kanamycin and 10% of human AB serum (to form a complete RPMI medium).

129

130 **Cell encapsulation to form 3-D culture microspheres**

131 Microspheres were generated as previously described (26, 32, 33). In short, to obtain a 3-D
132 culture, we re-suspended Mtb-infected host cells in complete RPMI medium and combined
133 with alginate-collagen matrix at 1 x 10^6 cells per ml. Microspheres were obtained by
134 introducing the sterile mix into the Electrostatic Bead Generator (Nisco, Zurich, Switzerland)
135 as described previously (34). Next, we equally distributed microspheres into 2ml
136 Eppendorfs, immersed them in 1ml of complete RPMI medium and kept at 37°C with 5%
137 CO_2. Bacterial bio-luminescence was observed with a GloMax® 20/20 Luminometer. In
138 order to carry out colony counts, Mtb was released from microspheres by dissolving in
139 55mM Sodium citrate / 10mM EDTA with 1% saponin in HBSS, pelleting at 10,000xg in a

140 Heraeus™ Pico™ microcentrifuge and plating onto 7H11 agar. Colony forming units were
141 counted at three weeks.
142

143 **Imaging by micro-focus X-ray Computed Tomography (μCT)**

144 μCT imaging was performed on the Nikon Med-X micro-CT scanner, optimised for
145 biomedically relevant low-contrast specimens. The equipment is supplied with a 130 kVp X-
146 ray source and a 2 x 2k flat panel detector allowing to obtain images at ~3μm spatial
147 resolution. Microspheres containing PBMCs infected with Mtb H37Rv were harvested at day
148 14 and prepared for imaging. Samples were fixed for 1 h at room temperature and then
149 overnight at 4°C in 3% glutaraldehyde in 0.1M cacodylate buffer (pH: 7.4) with an
150 osmolarity of 850 mOsm plus 2mM $CaCl_2$. Fixed samples were then washed with the 0.1M
151 cacodylate buffer (pH: 7.4), and post-fixed with 2% osmium tetroxide in 0.1M cacodylate
152 buffer (plus 2mM $CaCl_2$ and 2.3M sucrose). A sample was, then, treated with a single wash
153 step using a distilled water prior to staining it with 2% uranyl acetate (aq). Samples were
154 dehydrated through a series of ethanol submersion steps in 30%, 50%, 70%, 95%, 100%
155 alcohol solutions. The final 100% ethanol step was carried out twice. Next, the samples
156 were immersed in acetonitrile in order to facilitate the passage from ethanol to resin.
157 Samples were kept overnight in a 50:50 acetonitrile/ TAAB resin mixture. The next day, a
158 fresh resin was prepared and samples were incubated in it for further 6 h. Subsequently, the
159 samples were moved to embedding capsules with fresh TAAB resin. This resin block was
160 scanned with μCT. The 3-D stack generated had a voxel size of 2.7μm.

161 **3-D image processing**

162 On a specific Amira workstation, 3-D image segmentation and analysis were done using FEI
163 Amira software (version 6.4.0). In addition to the Wacom Cintiq 22HD touch-screen monitor
164 (resolution 1920x1080 pixels), the workstations include an Intel Xeon processor with 24
165 cores, 192 GB of RAM, and an Nvidia Quadro K4200 4GB graphics card. Manual
166 segmentation of microspheres containing aggregates (red), PBMC aggregates (blue), and
167 single PBMCs (yellow) was carried out on the 3-D dataset of the resin block containing
168 microspheres. 3-D reconstruction of the highlighted areas occurred using the 'Generate
169 Surface' feature of Amira software. A smoothing function was applied to all three different
170 labels to reduce the staircase effect. To visualise the result as a 3-D representation of the
171 manual segmentation, the 'Surface View' object was attached to the surface file.

172

173 **Scanning Electron Microscopy (SEM)**

174 Initial fixation and processing to the completion of ethanol drying was as above. The
175 microspheres were inserted in a metal holder with a grid after the final step of ethanol. Prior
176 to SEM imaging, the lid of the holder was shut and the entire holder put in an absolute
177 ethanol-filled glass vial. In order to achieve critical point drying, the samples were dried with
178 a Balzers CPD 030 drier. On a stub covered with two-sided tape, dried microspheres were
179 sprayed with gold and palladium (Pd), two non-oxidising metals, utilizing the Polaron E5100
180 sputter coater. This study was conducted with a FEI Quanta 200 Scanning Electron
181 Microscope for the acquisition of SEM images. Up to 30kV of power and 100,000x
182 magnification are available with this system. Ten kilovolts of power were used for
183 acquisitions.

184

185 **Eukaryotic Cell Viability/Toxicity Assays**

186 Microspheres were equally distributed into two 96-well plates and incubated at 37°C. At day
187 3 and 7, respectively, one plate was sacrificed for cell viability analysis. Promega's
188 CellTiter-Glo 3-D Cell Viability Assay was performed following the manufacturer's
189 specifications. Cellular necrosis in microspheres was measured using CytoTox-Glo
190 Cytotoxicity Assay (Promega). The GloMax® Discover 96 well plate reader (Promega, UK)
191 was used to monitor the luminescence. Additionally, cell toxicity was determined by
192 measuring the release of lactate dehydrogenase (LDH) with a colorimetric activity assay
193 (Roche, Burgess Hill, United Kingdom). The collected supernatants were stored at 4°C for
194 up to 7 days prior to analysis.

195

196 **Luminex analysis**

197 Supernatants were collected at day 3 and store at -20°C. Samples were sterilized by filtration
198 through a 0.22 mM Durapore membrane (Millipore) (35). We followed manufacturer's
199 protocol to determine concentrations of cytokines (Life Technologies, UK) and MMPs (R
200 and D Systems, UK) in the samples using a Bioplex 200 platform (Bio-Rad, Hemel
201 Hempstead, United Kingdom). Cytokine concentrations were measured using the cytokine
202 35-plex human panel (Thermofisher Scientific, UK). Analyses of MMP concentrations were
203 done by MMP fluorokine multianalyte profiling (R&D Systems, Abingdon, UK).

204

205 **Statistical Analysis**

206 All experiments were carried out on a minimum of two separate occasions from different
207 donors as biological replicates and each time with a minimum of three technical replicates.

208 Graph Pad Prism was utilized to carry out statistical analyses. Either ordinary one-way or 2-

209 way ANOVA Tukey's multiple comparisons tests were used to compare groups.

210

211

212 # Results

213 **Differential growth of Mtb strains in Middlebrook 7H9 broth versus 3-D microspheres**

214 To study Mtb proliferation in the context of host cells and extracellular matrix, we generated
215 3-dimensional microspheres, which consisted of human primary peripheral blood
216 mononuclear cells, Mtb and collagen (type I) using bio-electrospray methodology as
217 previously described (26, 27, 32, 33, 36). Scanning electron microscopy demonstrated the
218 external symmetry of the spheres (Fig. 1A). We characterised cellular aggregation within
219 spheres by micro-computed tomography (μ-CT), which demonstrated multicellular
220 granuloma formation at day 7 (Fig. 1B, C and Supplemental Video 1).

221 To monitor Mtb growth in our 3-D system, we generated luminescent clinical isolates by
222 incorporating the Lux operon (Table S1) (30). Microsphere luminescence closely correlates
223 with colony counts, permitting longitudinal analysis of growth within microspheres (32).
224 First, we compared Mtb proliferation in Middlebrook 7H9 broth to growth within
225 microspheres. The standard laboratory strain, H37Rv, grew significantly more rapidly in
226 broth culture than the clinical isolates ($p<0.0001$) (Fig. 1D). In contrast, in the 3-D system,
227 H37Rv was significantly attenuated in comparison to clinical isolates, which proliferated
228 much more rapidly over time (Fig. 1E). A similar pattern was observed when H37Rv was
229 compared with two other reference Mtb strains, CDC1551 and Erdman (Fig. S1). We then
230 repeatedly sub-cultured one of the clinical isolates of Beijing origin, 0414B, continuously in
231 7H9 broth for two years. This prolonged *in vitro* culture attenuated growth rate in infected
232 host cells in the 3-D model, to a similar extent to H37Rv, relative to freshly defrosted and
233 cultured 0414B (Fig. 1F). Therefore, the standard laboratory strain, H37Rv, is attenuated
234 compared to clinical isolates when analysed in a 3-D primary human cell culture model.

235

236 **Despite increased proliferation, the clinical strain is not cytotoxic and cytokine secretion**
237 **is suppressed**

238 Next, we investigated the effect of increased growth of the clinical isolate 0414B on host
239 cells in the 3-D system relative to the laboratory strain H37Rv. Host cells infected with
240 either Mtb strain survived better relative to uninfected PBMCs at day 3, analysed by two
241 different readouts, likely due to infection upregulation of pro-survival growth factors (Fig.
242 2A, B). However, we did not observe these differences using the Cell Titer Glo 3-D cell
243 viability assay at this time point (Fig. 2C). Comparing Mtb-infected cells, a significant
244 increase in toxicity with the clinical strain was only observed using CytoTox-Glo cytotoxicity
245 assay (Fig. 2A), not with the LDH assay or Cell Titer Glo 3-D cell viability assays at the 3-
246 day time point (Fig. 2B and C). There were some differences observed at the 7 day time
247 point, with the clinical strain causing greater toxicity as measured by LDH release (Fig. S2B).
248 These differences likely result from the technical features of the assays used, with LDH
249 released from dying cells and stable for 7 days, so this provides a slightly different measure
250 of cell death to the CytoTox-Glo cytotoxicity assay and Cell Titer-Glo assay. Later time
251 points did not reveal any significant differences between strains using the two luminescent
252 cell viability assays (Fig. S2A and C).

253

254 We compared the immunological response elicited by each strain at day 3, profiling secretion
255 of inflammatory mediators by Luminex array. Despite the significantly higher mycobacterial
256 load, secretion of pro-inflammatory cytokines was reduced from 0414B-infected
257 microspheres in comparison to the lab strain H37Rv (Fig. 3). Suppressed cytokine release
258 included TH_1 cytokines, TH_2 cytokines and some chemokines (Fig. 3 and Fig. S3). The
259 secretion of other analytes, such as IL-17A, IL-8, MCP-1 was upregulated by Mtb infection

260 equally in response to both strains, indicating that 0414B did not induce a global hypo-
261 secretory state. Similarly, secretion of the proteases matrix-metalloproteinase-1 (MMP-1)
262 and MMP-7 was equally upregulated by H37Rv and 0414B (Fig. 3 and Fig. S3).
263

264 **Clinical isolates are less susceptible to antibiotics in the 3-D system**

265 Having observed accelerated growth but reduced cytokine secretion for 0414B relative to
266 H37Rv in the microsphere system, we investigated relative efficacy of antibiotics against
267 each strain. Cell culture media around spheres were supplemented with standard first line
268 antibiotics: rifampicin, isoniazid, and pyrazinamide at physiological concentrations (1g/ml,
269 0.25g/ml and 500g/ml, respectively), on day 1 (32). The three-dimensional system
270 demonstrated inhibitory effects of all three antibiotics on H37Rv growth (Fig. 4A) as
271 previously reported, with luminescence falling progressively to baseline (32). In contrast,
272 after initial suppression, the clinical isolate 0414B regrew at day 12 despite incubation with
273 rifampicin and isoniazid (Fig. 4D). Furthermore, pyrazinamide was much less effective
274 against the clinical isolate relative to the laboratory strain, with complete killing of H37Rv
275 compared to temporary retardation of growth of 0414B. We tested a second clinical isolate,
276 1292F (lineage 4, Ural origin) with the four first-line drugs and once more observed
277 regrowth. Again, pyrazinamide was less effective (Table 1). Therefore, clinical isolates are
278 relatively more resistant to first line antibiotics in the 3-D model compared to the laboratory
279 strain H37Rv.
280

281 With the emergence of drug-resistant TB, novel and repurposed second-line antibiotics have
282 become increasingly important (2). Therefore, we analysed amikacin, moxifloxacin, D-
283 cycloserine and linezolid in the microsphere system. Moxifloxacin and linezolid (5µg/ml and

284 24μg/ml, respectively) completely inhibited the growth of all the drug-sensitive strains in the
285 3-D system (Fig. 4B, E, Table 1). Amikacin was also as effective (Fig. 4B, E and Table 1),
286 except for the Ural strain, 1292F, where the bacterial killing was relatively reduced (Table 1).
287 D-cycloserine was effective against H37Rv but minimally effective for the 0414B strain (Fig.
288 4E), but was efficacious against 1561Y and had intermediate activity against 1292F (Table
289 1). PAS (20μg/ml) had similar efficacy against all clinical isolates examined, partially
290 inhibiting growth (Table 1).
291
292 Finally, we tested emerging antibiotics bedaquiline and pretomanid (PA-824). Bedaquiline
293 effectively killed the laboratory strain H37Rv, but was significantly less efficacious against
294 the 0414B strain even at 10μg/ml (Fig. 4C, F). PA-824 completely inhibited growth of all
295 strains studied (Fig. 4C, F, Table 1). These studies further confirmed increased antibiotic
296 resistance in clinical strains relative to H37Rv.
297
298 **Effect of anti-TB drugs on MDR-TB growth in 3-D model**
299 Next, we investigated the efficacy of these antibiotics against MDR-TB in the 3-D model. As
300 a proof of principle, we selected two MDR-TB clinical isolates 1471A and 0940Y, both
301 belonging to the Beijing sub-lineage (Table S1), which have previously been characterised
302 phenotypically by standard assays and genotypically (11). Strain 1471A was resistant to
303 rifampicin, isoniazid and ethambutol, and strain 0940Y was resistant to rifampicin, isoniazid
304 and pyrazinamide based on standard individually validated liquid culture methodology. As
305 expected, rifampicin, isoniazid and ethambutol had no effect on 1471A growth, and
306 pyrazinamide had a moderate inhibitory effect (Fig. 5A). Again, the pyrazinamide efficacy
307 was reduced relative to H37Rv in the 3-D model. Strain 0940Y was resistant to rifampicin,
308 isoniazid and pyrazinamide, as anticipated. Unexpectedly, the strain also was resistant to

309 ethambutol in our system (Fig. 5D). Ethambutol is a bacteriostatic agent at the
310 concentrations used, and this may reflect the difficulty of reliable microbiological assays for
311 this drug.
312
313 From the second-line drugs investigated, only D-cycloserine at the lower concentration of
314 20µg/ml had no efficacy against the two strains in the 3-D system (Fig. 5B, E). Amikacin,
315 moxifloxacin and linezolid completely inhibited bacterial growth of both strains (Fig. 5B, C,
316 E, F; Table 1). PAS and PA-824 were similarly effective in bacterial killing as against the
317 laboratory strain H37Rv and for both MDR strains (Fig. 5 C, F and Table 1). Bedaquiline
318 (10µg/ml) had no effect inhibiting 1471A growth, while it moderately inhibited 0940Y
319 growth (Fig. 5C, F).
320
321 **Verapamil potentiates the effect of emerging antibiotics in the 3-D system**
322 These data demonstrate inherent antibiotic resistance in all Mtb clinical strains relative to
323 H37Rv, and confirm further additional resistance within the MDR strains, suggesting that
324 efflux pumps may be contributing to resistance and highlighting the need to investigate
325 adjunctive therapy to increase bactericidal activity (25). We focused on bedaquiline and
326 delamanid, important new MDR-TB drugs approved and recommended for treatment (2).
327 Delamanid is relatively understudied with a partially defined mechanism of action (37). In
328 7H9 broth, regrowth occurred after initial killing even at a relatively high concentrations of
329 delamanid (10µg/ml) (Fig. 6A, B). In contrast, delamanid fully inhibited bacterial growth in
330 the 3-D system (Fig. 6C, D), demonstrating greater efficacy in the microsphere system
331 relative to standard broth. The high delamanid concentrations required suggested efflux
332 pump activity contributed to antibiotic resistance, and so we investigated this further.
333

Verapamil, a Ca^{2+} channel blocker, potentiates the killing of mycobacteria with rifampicin, isoniazid (22-24) or bedaquiline (21, 38, 39). Consistent with these observations, we demonstrated that verapamil potentiated the effect of bedaquiline in the 3-D system in killing both laboratory and clinical strains (Fig. 7A, D). The effect was most marked for the clinical strain, changing a minor suppression of growth to rapid killing, and a similar effect was observed in 7H9 broth (Fig. S4A, B). Next, we investigated whether verapamil could also potentiate the effect of delamanid. Delamanid efficacy was augmented by verapamil in the 3-D system for the clinical isolate 0414B, preventing re-growth at late time points (Fig. 7B, C, E, F). In 7H9 broth, regrowth of both strains was delayed in the presence of verapamil together with delamanid, and this effect was greater for the clinical isolates (Fig. S4B, C, E, F).

Finally, we investigated whether other Ca^{2+} channel blockers and efflux pump inhibitors had a similar potentiating effect on delamanid as verapamil (Fig. S5A). In a host environment, verapamil is an inhibitor of broad-spectrum ABC transporter systems [e.g. P-glycoprotein (P-gp/ABCB1) also known as multidrug resistance protein 1 (MDR1)], and so we studied a P-gp specific, third-generation inhibitor, tariquidar, which is also known to inhibit Breast Cancer Resistance Protein (BCRP/ABCG2) (40). Tariquidar did not potentiate the effect of delamanid (0.5μg/ml) at concentrations ranging 0.5-50μg/ml (Fig. S5B). Similarly, diltiazem, a non-dihydropyridine (DHP) member of the calcium channel blocker class, had no effect (Fig. S5C), while amlodipine, which is a dihydropyridine (DHP) calcium channel blocker, had very minor potentiating effect on delamanid at 30μg/ml (Fig. S5D). Additionally, we investigated two compounds that act not only at the host level but also directly on mycobacteria. Chlorpromazine, a type II NADH dehydrogenase (NDH-2) inhibitor (41) affecting Mtb oxidative phosphorylation, had only minimal potentiating effect

359 and carbonyl cyanide m-chlorophenyl hydrazine (CCCP), a proton uncoupler (42, 43), had no
360 potentiating effect at the concentrations tested (Fig. S5E, F). Overall, none of the compounds
361 investigated exhibited a potentiating effect on delamanid that was as potent at that of
362 verapamil, suggesting verapamil has a unique mode of action in increasing antibiotic efficacy
363 in relation to bacteria interacting with cells of a host.

364 ## Discussion

365 Tuberculosis remains a depressingly persistent global pandemic, and drug resistance is an
366 increasing problem (2). Consequently, novel systems to evaluate antibiotic agents are needed
367 to identify alternative treatment approaches and combinations. Previously, the typical route
368 of identifying novel antibiotics was through the "three M's" route: minimal inhibitory
369 concentration (MIC), mouse and man (44), centred on enriched liquid culture media for MIC
370 and then minimum bactericidal activity (MBC) determination. Other more complex assays
371 are less frequently employed to identify lead compounds. Although the "3M" approach is
372 simple and straightforward, it does not incorporate the different physiological and anatomical
373 microenvironments of granulomas and cavities in a patient's lung (45, 46), and the effect of
374 these on drug penetration and activity. Consequently, this approach limits selection to
375 compounds that are most effective against rapidly replicating bacteria, potentially omitting
376 antibiotics that work against slowly replicating/dormant Mtb or are only efficacious in the
377 context of a combined drug-host immune response. However, the more complex the assays,
378 the more difficult, costly and lower the throughput for testing new compounds. In
379 considering the cost of clinical trials, there is significant scope to increase the initial
380 screening costs to save future expense; for example, a phase 3 novel antibiotic studies for
381 hospital–acquired bacterial pneumonia cost $89,600 per patient enrolled (47).

382 Consequently, better *in vitro* systems are required for TB drug discovery (48, 49). This
383 problem is illustrated by pyrazinamide, which would not have been identified through a
384 traditional strategy; PZA is not bactericidal under standard culture conditions and works
385 optimally under hypoxic conditions at pH 5.5 (50). Likewise, linezolid is bacteriostatic in
386 standard culture media, but a potent agent as part of combination treatment for M/XDR-TB
387 (2). The community is moving towards the concept of evaluating new combinations rather
388 than evaluation of single agent. The results of the STREAM 1 trial support this,

389 demonstrating that a short regimen (9-11 months) was non-inferior to a long regimen with
390 respect to the primary efficacy outcome and has a similar safety profile (15). We need to
391 evaluate novel drugs, and drug combinations, using different early stage models that can
392 identify agents that complement each other in their ability to penetrate the range of TB
393 lesions and to kill all bacterial sub-populations inside them (44). In addition, the bacterial
394 strains evaluated need to be standardised for reproducibility, and include clinically relevant
395 ones.

396 Mtb H37Rv has been a mainstay in TB research, but was isolated from a patient in the
397 beginning of 20th century and has been repeatedly sub-cultured since (51, 52). Here, our 3-D
398 granuloma model shows that relative to the standard laboratory H37Rv strain, the clinical
399 strain 0414B is more virulent, hypo-inflammatory and inherently antibiotic resistant. The co-
400 administration of verapamil increases the efficacy of bedaquiline and delamanid, and this
401 effect is most pronounced for clinical strains. Our findings further highlight the importance
402 of including diverse strains in drug discovery studies (7, 8, 52, 53), whilst supporting the
403 utility of advanced cell culture models incorporating human cells to refine the drug discovery
404 pipeline (48, 54).

405 Mtb strains H37Rv and 0414B belong to Mtb lineages 4 and 2, respectively. In Middlebrook
406 7H9 broth, H37Rv grew much better than other strains investigated belonging to lineages 2
407 (‘modern’ Beijing strains) and lineage 4 (reference strains: Erdman and CDC1551), which
408 has been observed previously (55), but conversely was significantly attenuated in the
409 microspheres system compared to these strains. In experiments with mice, lineage 2 strains
410 produced high levels of bacilli in their lungs (56). Furthermore, studies using 2-D human cell
411 culture systems demonstrated that mycobacteria of this genotype replicate more rapidly in
412 comparison to the laboratory reference strains (57-59), and that lineage 2 strains spread more

413 rapidly between cells than H37Rv (60), supporting our observation of increased
414 pathogenicity.

415 Increased proliferation of the clinical strain was not associated with greater host cytotoxicity,
416 suggesting Mtb accumulates within host cells without destroying them. H37Rv was hyper-
417 inflammatory in contrast to the clinical isolates, inducing less cytokine secretion. Diverse
418 human and animal evidence suggests that the enhanced virulence of the Beijing strains is
419 partially due to induction of lower levels of Th1 cytokines such as TNF-α, IL-6, IL-10, IL-12
420 and IFN-γ (55, 58, 61-66). We did not observe any significant difference in the expression of
421 MCP-1 between the Beijing strain and laboratory strain, which has been shown previously
422 (61), and did not detect difference in the expression of IFN-α. It has been suggested that
423 Beijing strains induce more type 1 interferons (67, 68), however, other studies have shown
424 that IFN-α equalizes in lungs of BALB/c mice by day 14 (56), consistent with our findings.
425 Therefore, differences in the cytokine induction between strains clearly depends on the model
426 system studied.

427 We have previously shown that Mtb is pyrazinamide sensitive in the 3D model but not
428 standard 2-D culture (29). Here, antibiotic sensitivity testing in the 3-D model showed that
429 clinical isolates are significantly less susceptible to drugs than the laboratory strain, H37Rv.
430 Bedaquiline (10μg/ml) was ineffective against the 1471A strain and only slightly effective
431 for 0940Y clinical isolate, and bedaquiline resistance is emerging (69-72). This is likely due
432 to genotypical differences between the MDR strains. Sequencing of the strains investigated
433 revealed non-synonymous single nucleotide polymorphism (ns-SNP) differences in multiple
434 genes including efflux pumps and genes involved in respiration between the clinical isolates
435 and H37Rv (11). Whether this contributes to increased drug resistance of the clinical strains
436 needs to be determined (73-75). Evidently, the divergence between results from traditional
437 sensitivity testing methods and advanced cell culture systems has widespread clinical

438 implications, as *in vivo* Mtb is primarily within host phagocytes and under a stressed
439 environment. For example, a synergistic effect between pyrazinamide and bedaquiline was
440 recently highlighted by advanced imaging studies of infected human cells (50).

441 We investigated the response to emerging antibiotics bedaquiline and delamanid between the
442 clinical isolate and the laboratory strain and explored the potential of verapamil in increasing
443 efficacy. We observed that verapamil potentiates the killing effect of bedaquiline in our
444 microsphere system, which has been previously reported in other models (25, 38, 39). We
445 then showed that the effect of delamanid can similarly be potentiated by verapamil, although
446 the effect was less marked. Verapamil augmentation for each antibiotic was greater for the
447 clinical strain in the 3-D system, indicating strain-specific differences. Verapamil may result
448 in a cascade of events involving the inhibition of respiratory chain complexes and energy
449 production for efflux production in mycobacteria, and therefore the effect potentiating anti-
450 TB drugs is indirectly increased (24). Verapamil's mechanism of action has been proposed
451 to be through affecting the membrane energetics of *M. tuberculosis* (76) rather than a direct
452 bacterial efflux pump inhibition as previously believed (23, 24, 38, 39), potentially explaining
453 why other channel blockers were ineffective in our system. Overall, the potentiating effect of
454 verapamil on the efficacy of antibiotics within the 3-D system was marked, in particular
455 when clinical strains were studied.

456 Investigation of calcium channel blockers and efflux pump inhibitors other than verapamil
457 had no combined effect with delamanid. Within our 3-D cell culture model, transport
458 systems will be active on three separate membranes: the mycobacterial membrane, the
459 phagolysosomal membrane and the macrophage cell membrane, representing a significant
460 experimental challenge to fully dissect underlying mechanisms. At the same time, this
461 reflects the true complexity of events in patients. We suspect that verapamil may be

462 enhancing the potency of delamanid by acting at all three membranes, and this additive effect
463 leads to the overall phenotype.

464 In conclusion, we demonstrate that clinical and laboratory strains have significantly different
465 virulence and antibiotic sensitivity when studied in a human 3-D cell culture system.
466 Prolonged subculture of a clinical strain leads to attenuation, demonstrating that the system
467 can be utilised to investigate mycobacterial pathogenic factors. Our data demonstrate that the
468 3-D bioelectrospray model, where gene expression reflects events in patients (27) and mirrors
469 the critical 3D granuloma organisation (46, 77), is an additional platform to test the
470 effectiveness of antibiotics and identifies differences in antibiotic sensitivity and the effect of
471 adjunctive therapy. Studying more complex assays at the preliminary drug development
472 stage may help identify and refine optimal lead combinations.

473 **Conflict of interest:** The authors declare that the research was conducted in the absence of
474 any commercial or financial relationships that could be construed as a potential conflict of
475 interest.

476

477 **Author contributions**: MK, LT and EK performed the laboratory analyses, NC and XG
478 characterized the Mtb strains, OK supervised the micro-CT analyses, FD and PE conceived
479 the project and coordinated the experiments. All authors had intellectual input into the
480 progression of the project and contributed to the manuscript writing.

481

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	Strain / Abx (µg/ml)	RIF 1	INH 0.25	PZA 500	EMB 4	AMIK 15	MOXI 5	DCS 20	LNZ 24	BDQ 10	PAS 20	PA-824 3
Lab	H37Rv lux	+	+	+	+	+	+	+	+	+	+/-	+
Sensitive	1292F lux	+	+	+/-	+	+/-	+	-/+	+	+	+/-	+
	1561Y lux	+	+	+/-	+	+	+	+	+	+/-	+/-	+
	0414B lux	+	+	-/+	+	+	+	-	+	-/+	+/-	+
MDR	1471A lux	-	-	-/+	-	+	+	-	+	-	+/-	+
	0940Y lux	-	-	-	-	+	+	-	+	-/+	+/-	+

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498 **Table 1: Summary of efficacy of antibiotics against different clinical isolates in the 3-D**

499 **model.** RIF - rifampicin; INH - isoniazid; PZA - pyrazinamide; EMB - ethambutol; AMIK -

500 amikacin; MOXI - moxifloxacin; DCS - D-cycloserine; LNZ - linezolid; BDQ - bedaquiline;

501 PAS - para-aminosalicylic acid; PA-824 - pretomanid.

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506 ## Figure legends

507

508 **Fig. 1: Mycobacterial clinical isolates grow more rapidly in the 3-D microsphere model. (A)**
509 Scanning electron microscopy of microspheres to demonstrate appearance. **(B, C)** Micro-CT scan
510 (μCT) of embedded microspheres with 3-D reconstruction. Spheres appear different sizes as different
511 levels have been captured within the block. (B) External appearance and cut surface. (C) Saggital
512 section. Representative microspheres were marked in red, showing equal distribution of PBMCs
513 within microsphere (yellow). A cellular aggregate is visible in the centre sphere (blue). **(D)** H37Rv
514 lux grows faster than clinical isolates (1561Y lux, 0414B lux) and MDR-TB strains (1471A lux,
515 0940Y lux) in Middlebrook 7H9 broth measured by optical density (OD) at 600nm. Experiments
516 were performed in triplicate. **(E)** Conversely, H37Rv lux growth in the 3-D system is attenuated in
517 comparison to clinical isolates measured by luminescence. **(F)** Beijing lineage clinical isolate 0414B
518 lux becomes attenauated after continuous 2-year culturing in broth when studied in the microsphere
519 system, growing at a similar rate to the lab strain H37Rv lux, in contrast to freshly defrosted 0414B
520 lux strain. **(E, F)** Data are the mean ± SEM of an experiment performed in triplicate and are
521 representative of two separate experiments. Statistics: 2way ANOVA Tukey's multiple comparisons
522 test **(D,E,F)**; **** $p<0.0001$.

523 **Fig. 2: Host cells infected with Mtb H37Rv or a clinical strain have similar survival despite**
524 **differences in bacterial proliferation.** After infection with H37Rv lux (black) or 0414B lux (red),
525 cells survived better than uninfected cells (A, B). A minimal difference was observed in relative host
526 cell toxicity betweeen strains measured by CytoTox-Glo cytotoxicity assay (A), but no significant
527 differences were seen with LDH cytotoxicity assay (B) and Cell Titer-Glo 3-D cell viability assay (C)
528 between strains at day 3. Data are mean +/- SEM of 3 separate experiments. Statistics: Ordinary one-
529 way ANOVA with Tukey's multiple comparisons test **(A, B, C)**; **** $p<0.0001$, ** $p<0.01$, * $p<0.1$,
530 ns $p>0.05$.

531 **Fig. 3: The clinical Mtb strain is less pro-inflammatory than 0414B.** A heatmap of differences in
532 cytokine secretion by PBMCs within microspheres after infection either with the laboratory strain

533 H37Rv lux or the clinical isolate 0414B lux, measured at day 3 by Luminex array. Cytokine secretion
534 was normalised to concentration of H37Rv-infected cells (data are presented as a percentage
535 normalised to H37Rv lux strain). Secretion of thirteen analytes was significantly reduced for the
536 clinical strain in contrast to the lab strain, despite greater bacterial proliferation. Other analytes, such
537 as IL-17A, MCP-1, MMP-1 and MMP-7, were equally upregulated by Mtb infection. Control:
538 Uninfected PBMCs within microspheres. Normalised data from two donors analysed in triplicate are
539 presented.

540 **Fig. 4: The 0414B clinical strain is antibiotic resistant relative to Mtb H37Rv.** Antibiotics were
541 added at day 1 to the 3-D system and Mtb growth measured with luminescence; Standard drugs:
542 rifampicin (RIF, 1µg/ml, dark red), isoniazid (INH, 0.25µg/ml, navy blue) and pyrazinamide (PZA,
543 500µg/ml; dark green); second-line drugs: amikacin (AMIK, 15µg/ml, mint green), moxifloxacin
544 (MOXI, 5µg/ml, light brown) and D-cycloserine (DCS, 20µg/ml, lilac); emerging drugs: bedaquiline
545 (BDQ, 10µg/ml, dark brown) and pretomanid (PA-824, 3µg/ml, light blue). H37Rv lux growth was
546 inhibited by all antibiotics tested (A, B and C). The clinical isolate 0414B regrew at day 12 despite
547 incubation with RIF and INH, and PZA was much less effective against the clinical isolate relative to
548 the laboratory strain (D). From the second-line antibiotics, amikacin and moxifloxacin completely
549 inhibited the clinical isolate's growth but D-cycloserine had only a minor effect (E). Similarly,
550 bedaquiline had only a partial effect in contrast to PA-824, which was very effective against 0414B
551 lux (F). Dimethyl sulfoxide (DMSO); used as solvent for rifampicin, bedaquiline and pretomanid in
552 the control sample did not affect the growth of Mtb. Background level of luminescence is designated
553 by crosses (x). Black arrow specifies antibitotic addition. The experiment was performed in triplicate
554 on two separate donors, and a representative experiment is shown. Statistical analyses were done
555 using 2way ANOVA Tukey's multiple comparisons test; **** $p<0.0001$. The data are very
556 consistent, so SEM bars are very narrow and obscured.

557 **Fig. 5: Multi-drug resistant (MDR) strains demonstrate extensive antibiotic resistance.**
558 Antibiotics were added at day 1 to the 3-D model and Mtb growth measured with luminescence; first-
559 line drugs: rifampicin at 1µg/ml (RIF 1, dark red), isoniazid at 0.25µg/ml (INH 0.25, navy blue),

560 pyrazinamide at 500µg/ml (PZA 500, dark green) and ethambutol at 4µg/ml (EMB 4, orange);
561 second-line drugs: moxifloxacin at 5µg/ml (MOXI 5, light brown), D-cycloserine at 20µg/ml (DCS
562 20, lilac) and 200µg/ml (DCS 200, dark purple) and linezolid at 24µg/ml (LNZ 24, mid-purple);
563 emerging drugs: bedaquiline at 5µg/ml (BDQ 5, beige) and 10µg/ml (BDQ 10, dark brown); and
564 pretomanid at 3µg/ml (PA-824 3, light blue). 1471A lux strain confirmed resistance to rifampicin,
565 isoniazid and ethambutol and showed partial sensitivity to pyrazinamide (A). Growth of this multi-
566 drug resistant clinical isolate was unaffected by the lower concentration of D-cycloserine and
567 bedaquiline (both concentrations tested) (B, C). 0940Y lux strain was resistant to all first-line
568 antibiotics (D) and D-cycloserine at the lower concentration (E). 0940Y had partial sensitivity to
569 bedaquiline (F). Bacteria were fully inhibited by all other antibiotics examined. Mtb growth was
570 unaltered by adding DMSO, used as solvent for rifampicin, linezolid, bedaquiline and PA-824.
571 Crosses (x) designate background level of luminescence. Black arrow shows antibitotic addition.
572 The experiment was performed in triplicate on two separate donors, and a representative experiment is
573 shown. Statistical analyses were carried out using 2-way ANOVA with Tukey's multiple
574 comparisons test; **** $p<0.0001$.

575 **Fig. 6: Delamanid is more effective in microspheres than 7H9 broth.** Delamanid was added at day
576 1 to either 7H9 broth (A, B) or infected microspheres (C, D) and Mtb growth monitored by
577 luminescence. Bacteria were inhibited to the background level with delamanid at 10µg/ml (DLM 10,
578 khaki green) in the microsphere system (C, D). Conversely, delamanid in 7H9 broth was much less
579 effective in killing either H37Rv or O414B (A, B). Mtb growth was unaffected by DMSO, used as
580 solvent for delamanid. Background level of luminescence is shown by crosses (x). Adding of
581 antibiotics is marked by a black arrow. The experiment was performed in triplicate on two separate
582 donors, and a representative experiment is shown. Statistical analyses were performed using 2-way
583 ANOVA with Tukey's multiple comparisons test; **** $p<0.0001$, *** $p<0.001$.

584 **Fig. 7: Verapamil increases efficacy of bedaqualine and delamanid in 3-D culture.** Compounds
585 were supplied at day 1 to the 3-D system and Mtb growth observed by luminescence; bedaquiline at
586 5µg/ml (BDQ 5, beige) and 10µg/ml (BDQ 10, dark brown); verapamil at 50µg/ml (VPL 50, light

587 blue); delamanid at 0.5µg/ml (DLM 0.5, orange) and 5µg/ml (DLM 5, light green). Verapamil alone
588 slightly reduced the growth of Mtb in all conditions. Verapamil potentiated the effect of bedaquiline
589 for both H37Rv lux and 0414B lux in the 3-D system (A, D). Delamanid at 0.5µg/ml combined with
590 verapamil had a greater killing effect on the clinical isolate than on H37Rv (B, E). Delamanid at the
591 higher concentration inhibited the bacterial growth to the background level (C, F). Slight re-growth
592 was observed at a later time point for 0414B lux, which was inhibited when combined with verapamil.
593 Mtb growth was unaffected by DMSO, used as solvent for all the compounds tested. Crosses (x)
594 show background level of luminescence. Black arrow points to antibitotic addition. The experiment
595 was performed in triplicate on two separate donors, and a representative experiment is shown.
596 Statistics: 2way ANOVA Tukey's multiple comparisons test; **** $p<0.0001$, ** $p<0.01$, ns $p>0.05$

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603 ## Supplementary materials

604 **Fig. S1: Clinical isolate 0414B lux and other reference strains grow more rapidly in the 3-D**
605 **model. (A)** H37Rv lux grows faster than the clinical isolate 0414B lux and investigated reference
606 strains (Erdman lux and CDC1551 lux) in Middlebrook 7H9 broth measured by optical density (OD)
607 at 600nm. Experiments were performed in triplicate. **(B)** H37Rv lux is attenuated in comparison to
608 the clinical isolate 0414B lux and the reference strains, Erdman lux and CDC1551 lux, in the 3-D
609 system measured by luminescence. **(C)** Colony counts performed at day 26 confirm that H37Rv lux
610 growth is reduced in comparison to the clinical isolate 0414B lux and the reference strains, Erdman
611 lux and CDC1551 lux, in the 3-D system. Colour code as in (A) and (B). (B, C) Data are the mean ±
612 the standard error of the mean of an experiment performed in triplicate and are representative of two
613 separate experiments. Statistical analyses were carried out using 2-way ANOVA with Tukey's
614 multiple comparisons test (A, B) or Ordinary one-way ANOVA Tukey's multiple comparisons test
615 (C); **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$.

616 **Fig. S2: Survival of host cells at day 7.** Upon infection with either H37Rv lux (black), reduced
617 cytotoxicity compared to uninfected cells was noted by LDH release and Cell Titer-Glo 3-D cell
618 viability assay. When compared to 0414B lux (red), no difference was observed in host cell toxicity
619 measured by CytoTox-Glo cytotoxicity assay (A) or Cell Titer-Glo 3-D cell viability assay (C)
620 between strains. Toxicity of host cells infected with H37Rv lux was significantly lower than cells
621 infected with clinical isolate 0414B lux at day 7 when analysed by the LDH cytotoxicity assay (B).
622 Data are mean +/- SEM of 3 separate experiments. Statistics: Ordinary one-way ANOVA with
623 Tukey's multiple comparisons test (A,B,C); *** $p<0.001$, * $p<0.1$, ns $p>0.05$.

624 **Fig. S3: Secretion of multiple cytokines by 0414B-infected cells is reduced relative to H37Rv**
625 **infected cells.** The clinical isolate 0414B lux was significantly less pro-inflammatory than the
626 laboratory strain H37Rv lux. Panels A, B, C, D, E and F show six significantly different cytokines in
627 response to the two strains. Panels G, H, I, J, K and L illustrate cytokines and MMPs that were
628 secretion was similary upregulated in response to the two strains, analysed in the same samples by
629 Luminex multiplex array. Uninfected PBMCs are control. Normalised data from two donors

630 analysed in triplicate are presented (data are presented as a percentage vs H37Rv lux strain).
631 Statistical analyses were performed using Ordinary one-way ANOVA with Tukey's multiple
632 comparisons test; **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.1$, ns $p>0.05$.

633 **Fig. S4: Verapamil increases Mtb killing in 7H9 broth.** Anti-TB drugs were added at day 1 to 7H9
634 broth and Mtb growth monitored by luminescence; bedaquiline at 5µg/ml (BDQ 5, beige) and
635 10µg/ml (BDQ 10, dark brown); verapamil at 50µg/ml (VPL 50, light blue); delamanid at 0.5µg/ml
636 (DLM 0.5, orange) and 5µg/ml (DLM 5, light green). Verapamil alone did not affect initial growth
637 of Mtb, and had a minimal inhibitory effect at later time points (A, B, C, D, E, F). Bedaquiline was
638 more effective against the clinical isolate than the laboratory strain (A, B). Verapamil's potentiating
639 effect on bedaquiline in killing bacteria was observed at later time points for H37Rv lux (A) and
640 minimally 0414B lux (B). Delamanid had substantial killing effect on Mtb, however bacteria quickly
641 recovered (B, C, E, F). The decrease in bacterial growth was potentiated by verapamil and it was
642 considerably greater for the clinical isolate relative to H37Rv, but bacteria revived at later time points
643 (B, C, E, F). Mtb growth was unaffected by DMSO, used as solvent for all the compounds tested.
644 Crosses (x) show background level of luminescence. Adding of antibiotics is specified by a black
645 arrow. Data are mean +/- SEM for an experiment performed in triplicate and representative of 2
646 separate experiments. Statistical analyses were done using 2-way ANOVA with Tukey's multiple
647 comparisons test; **** $p<0.0001$, * $p<0.1$, ns $p>0.05$.

648 **Fig. S5: Alternative efflux pump inhibitors combined with delamanid do not have as potent an**
649 **effect as verapamil in the 3-D system.** Compounds were added at day 1 to 3-D culture and Mtb
650 growth monitored by luminescence; delamanid at 0.5µg/ml (DLM 0.5, orange); verapamil at 50µg/ml
651 (VPL 50, light blue); tariquidar at 0.5µg/ml (TQD 0.5, light grey-green), 5µg/ml (TQD 5, light
652 brown), 10µg/ml (TQD 10, dark red) and 50µg/ml (TQD 50, dark brown); diltiazem at 5µg/ml (DLZ
653 5, light blue) and 50µg/ml (DLZ 50, mid-blue); amlodipine at 3µg/ml (AML 3, bright red) and
654 30µg/ml (AML 30, dark red); chlorpromazine at 2µg/ml (CPZ 2, light green) and 20µg/ml (CPZ 20,
655 dark green); carbonyl cyanide 3-chlorophenylhydrazone at 2µg/ml (CCCP 2, lilac) and 20µg/ml
656 (CCCP 20, mid-purple). Verapamil showed a potentiating effect on delamanid in inhibiting Mtb

657 growth (A). Addition of tariquidar, diltiazem or CCCP to delamanid had no effect at the
658 concentrations tested (B, C, F). Supplementation of amlodipine with delamanid had minimal effect
659 but only at a higher concentration used (D). Similarly, combining chlorpromazine 20µg/ml with
660 delamanid showed very slight Mtb killing effect (E). Higher concentrations of tariquidar and
661 chlorpromazine applied on their own (50µg/ml and 20µg/ml, respectively) had some inhibitory effect
662 on the clinical isolate (B, E). Mtb growth was unaffected by DMSO, used as solvent for all the
663 compounds tested. Crosses (x) represent background level of luminescence. Black arrow shows
664 antibitotic addition. Data are mean +/- SEM for an experiment performed in triplicate and
665 representative of 2 separate experiments. Statistical analyses were carried out using 2-way ANOVA
666 with Tukey's multiple comparisons test; ns $p>0.05$

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669 **Supplementary video 1:** Reconstruction of micro-CT images of Mtb-infected microspheres
670 embedded in resin. For illustrative purposes, 3 microspheres have been highlighted, with
671 cells within spheres highlighted yellow and cellular aggregates blue. Available for review at:
672 https://www.dropbox.com/s/kcnooi7pj0xbibl/Microsphere_video_100719.mp4?dl=0

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