

## A multi-targeting pre-clinical candidate against drug-resistant tuberculosis

Kaur, Parvinder; Potluri, Vijay; Ahuja, Vijay Kamal; Naveenkumar, C N; Krishnamurthy, Ramya Vadageri; Gangadharaiah, Shruthi Thimmalapura; Shivarudraiah, Prasad; Eswaran, Sumesh; Nirmal, Christy Rosaline; Mahizhaveni, Balasubramanian; Dusthacker, Azger; Mondal, Rajesh; Batt, Sarah M; Richardson, Emily J; Loman, Nicholas J; Besra, Gurdyal Singh; Shandil, Radha Krishan; Narayanan, Shridhar

DOI:  
[10.1016/j.tube.2021.102104](https://doi.org/10.1016/j.tube.2021.102104)

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*Document Version*  
Peer reviewed version

*Citation for published version (Harvard):*  
Kaur, P, Potluri, V, Ahuja, VK, Naveenkumar, CN, Krishnamurthy, RV, Gangadharaiah, ST, Shivarudraiah, P, Eswaran, S, Nirmal, CR, Mahizhaveni, B, Dusthacker, A, Mondal, R, Batt, SM, Richardson, EJ, Loman, NJ, Besra, GS, Shandil, RK & Narayanan, S 2021, 'A multi-targeting pre-clinical candidate against drug-resistant tuberculosis', *Tuberculosis*, vol. 129, 102104. <https://doi.org/10.1016/j.tube.2021.102104>

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## 1           **A Multi-Targeting Pre-Clinical Candidate against Drug-Resistant Tuberculosis**

2   \*Parvinder Kaur<sup>a</sup>, Vijay Potluri<sup>a</sup>, Vijay Kamal Ahuja<sup>a</sup>, C.N.Naveenkumar<sup>a</sup>, Ramya Vadageri  
3   Krishnamurthy<sup>a</sup>. Shruthi Thimmalapura Gangadharaiah<sup>b</sup>, Prasad Shivarudraiah<sup>b</sup>, Sumesh Eswaran<sup>b</sup>,  
4   Christy Rosaline Nirmal<sup>c</sup>, Balasubramanian Mahizhaveni<sup>c</sup>, Azger Dusthacker<sup>c</sup>, Rajesh Mondal<sup>c</sup>, Sarah  
5   M. Batt<sup>d</sup>, Emily J. Richardson<sup>d</sup>, Nicholas J. Loman<sup>d</sup>, Gurdyal Singh Besra<sup>d</sup>, Radha Krishan Shandil<sup>a</sup>,  
6   Shridhar Narayanan<sup>a</sup>.

7   <sup>a</sup>Foundation for Neglected Disease Research, Bangalore, India.

8   <sup>b</sup>Anthem BioSciences. Pvt. Ltd., No 49, Canara Bank Road, Hosur Rd, Electronics City Phase 1,  
9   Bommasandra Industrial Area, Bengaluru, Karnataka 560099, India.

10   <sup>c</sup>National Institute for Research in Tuberculosis, No.1, Mayor Sathiyamoorthy St, Chetpet, Chennai,  
11   Tamil Nadu 600031, India.

12   <sup>d</sup>Institute of Microbiology & Infection, School of Biosciences, University of Birmingham, Edgbaston,  
13   Birmingham B15 2TT, UK.

14   \*[parvinder.kaur@fndr.in](mailto:parvinder.kaur@fndr.in). Foundation for Neglected Diseases Research (FNDR), Plot 20A, KIADB  
15   Industrial Area, Veerapura, Doddaballapur, Bangalore – 561203, Karnataka, India.

### 17   **Abstract**

18   FNDR-20081 [4-{4-[5-(4-Isopropyl-phenyl)-[1,2,4]oxadiazol-3-ylmethyl]-piperazin-1-yl}-7-pyridin-  
19   3-yl-quinoline] is a novel, first in class anti-tubercular pre-clinical candidate against sensitive and drug-  
20   resistant *Mycobacterium tuberculosis* (Mtb). *In-vitro* combination studies of FNDR-20081 with first-  
21   and second-line drugs exhibited no antagonism, suggesting its compatibility for developing new  
22   combination-regimens. FNDR-20081, which is non-toxic with no CYP3A4 liability, demonstrated  
23   exposure-dependent killing of replicating-Mtb, as well as the non-replicating-Mtb, and efficacy in a  
24   mouse model of infection. Whole genome sequencing (WGS) of FNDR-20081 resistant mutants  
25   revealed the identification of pleotropic targets: *marR* (Rv0678), a regulator of MmpL5, a  
26   transporter/efflux pump mechanism for drug resistance; and Rv3683, a putative metalloprotease  
27   potentially involved in peptidoglycan biosynthesis. In summary, FNDR-20081 is a promising first in  
28   class compound with the potential to form a new combination regimen for MDR-TB treatment.

30 **Keywords**

31 Drug resistance, *Mycobacterium tuberculosis*, first-in-class, multi-target, pre-clinical candidate.

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## 51 Introduction

52 Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a communicable disease that is a major  
53 cause of ill health, one of the top 10 causes of death worldwide and the leading cause of death from a  
54 single infectious agent (ranking above HIV/AIDS) [1]. Despite notable progress to control TB, in 2019,  
55 globally an estimated 10 million people developed TB, 1.4 million died and about 0.4 million of these  
56 cases were due to HIV co-infection [1]. Furthermore, the TB drug-resistance is increasing globally and  
57 has limited treatment options [1]. There are approximately 206 030 reported cases [1] representing multi-  
58 drug resistant TB (MDR/RR-TB) in 2019, with most cases attributed geographically to India (24%);  
59 China (13%) and the Russian Federation (10%). Additionally, 5.99% of cases were reported as  
60 extensively drug-resistant TB (XDR-TB). The ‘End TB Strategy’ aims to reducing TB deaths and  
61 incidence by 2025 [2]. But the global incidence and mortality rates have fallen by a mere 2- 3% each  
62 year. Hence, to achieve these targets, we need novel bactericidal anti-TB drugs, efficacious against  
63 replicating and, non-replicating populations, as well as against drug resistant TB [1,3,4].

64 About a quarter of the global population is latently infected with *Mycobacterium tuberculosis* (Mtb),  
65 and prone to develop active TB disease during their lifetime if immunocompromised [1]. Thus, latent-  
66 TB can seriously skew the treatment logistics and strategies. Hence, novel compounds with activity  
67 against non-replicating populations (NRP) of Mtb must be developed as a priority. Few new anti-TB  
68 drugs have reached the stage of clinical development and use in patients after a gap of 50 years  
69 (Bedaquiline, Delamanid, Pretomanid (PA824)), while a few more are in the pipeline e.g. Q203, TBA-  
70 7371 etc. [5,6]. These compounds offer hope that new drugs hitting novel targets in Mtb could be  
71 successfully developed for the treatment of TB.

72 The discovery of new chemical scaffolds with novel mechanism of action, are necessary to develop  
73 improved therapeutic combinations for the treatment of MDR-TB. We synthesized and screened [7] a  
74 small molecule library of quinoline derivatives (Figure 1) and identified a potent inhibitor FNDR-20081  
75 [8], against *M. tuberculosis* H37Rv with an MIC of 0.5-2 µg/mL. FNDR-20081 is a 1<sup>st</sup> in class novel  
76 drug-like molecule, highly TB-specific, non-cytotoxic (IC<sub>50</sub> >100µM) on THP-1 and HepG2 cells.  
77 FNDR-20081 is active *in-vitro* against sensitive and MDR TB clinical isolates, exhibited no adverse  
78 drug-drug interactions with first- and second-line anti-TB drugs *in-vitro*, orally bioavailable and showed  
79 *in-vivo* efficacy. FNDR-20081 represents a potential anti-tubercular candidate to develop novel

80 combinations with existing drugs and new compounds that may become clinically relevant in the  
81 treatment of MDR TB.

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## 101 **Materials and methods**

102 **Drugs, Chemicals and Media used:** Reference antibiotics Streptomycin (STR), Isoniazid (INH),  
103 Rifampicin (RIF), Ethambutol (EMB), Amikacin (AMK), Capreomycin (CAP), Kanamycin (KAN), D-  
104 cycloserine (DCS), Clofazimine (CLO) and Fluoroquinolones (FQs) were obtained from Sigma-Aldrich  
105 (Merck USA). Media and the supplements used in this study were Middlebrook 7H11 Agar base,  
106 Middlebrook 7H9 broth base and ADC (albumin, dextrose, and catalase) supplements (BD/Difco),  
107 Tween-80 (Merck-SIGMA). The stock solutions (12.8 mg/mL) of test compounds and the reference  
108 drug controls (e.g., RIF) were prepared separately in dimethyl sulfoxide (DMSO) or in Milli-Q water  
109 (e.g., INH) as appropriate. Working solutions were freshly prepared at the time of experiment.

110 **Bacterial strains:** *M. tuberculosis* H37Rv (WT) and a total of 61 different reference strains (ATCC),  
111 and clinical isolates (National Institute for Research in Tuberculosis, NIRT Chennai) of Mtb, as well as  
112 *Mycobacterium bovis* (*M. bovis*) BCG Pasteur strains (University of Birmingham) were used in this  
113 study. Mtb cultures were sub-cultured and grown in Middlebrook 7H9 broth supplemented with 10%  
114 (v/v) Middlebrook ADC, 0.05% (v/v) Tween-80 and 0.25% (v/v) glycerol) to a cell density of  $10^9$   
115 colony-forming units (CFU)/mL. Glycerol stocks were prepared and stored at  $-80^{\circ}\text{C}$  in 0.5ml aliquots.  
116 A single vial was thawed and used each time for each experiment. Apart from Mtb, the compounds were  
117 profiled against another bacterial non-TB panel or ESKAPE panel (*Enterococcus faecium* [VRE],  
118 *Staphylococcus aureus* [MRSA], *Klebsiella pneumoniae* [sensitive], *Acinetobacter baumannii*  
119 [sensitive], *Pseudomonas aeruginosa* [sensitive], *Enterobacter aerogenes* [sensitive]) as well. This  
120 study was approved by the institutional bio-safety committee (IBSC).

121 **MIC determination in *M. tuberculosis* WT and *M. bovis* BCG, as well as clinical isolates:** Minimum  
122 inhibitory concentrations (MICs) were determined against Mtb strains by the standard broth dilution  
123 method according to CLSI guidelines M24 [9,10,11]. Briefly, the test compounds were dissolved in  
124 DMSO, serially diluted by 2-fold in a 10-concentration dose response (10c-DR) ranging from 128 to  
125  $0.25\ \mu\text{g}/\text{mL}$  in 96-well plates. Middlebrook 7H9 broth (supplemented with 10% ADC) complete media  
126 was used for the assay. Mtb culture was added as  $200\ \mu\text{L}$  in each well to all columns except the media  
127 control column ( $200\ \mu\text{L}$  of media was added) to give a final inoculum of  $3-7 \times 10^5$  cfu/mL. The quality  
128 control (QC) included: media controls, growth controls (including DMSO controls), and the reference  
129 drug inhibitors (Rifampicin and Isoniazid). The assay plates were incubated at  $37^{\circ}\text{C}$ , resazurin dye was  
130 added on 6th day, and the results were noted on the 7<sup>th</sup> day as colorimetric readout. The blue wells

131 indicated inhibition of growth, while the pink wells indicated uninhibited growth. The MIC was defined  
132 as the minimum concentration that completely inhibited the growth of bacteria. MIC assays were carried  
133 out in duplicate.

134 The MIC values of FNDR-20081 for a total of 61 Mtb clinical isolates were evaluated. These clinical  
135 isolates comprised of XDR (5), MDR (33), SDR (15), drug-sensitive (8) Mtb strains. The definition of  
136 SDR, MDR, and XDR is as follows: SDR= Resistance to any single drug (INH, RIF, STR, EMB, PAS,  
137 AMK, KAN, CAP, OFX, MXF) MDR=Resistance to any 2 or more drugs of the above mentioned,  
138 XDR= Resistance to all 1st line drugs + 2nd line+ 1 injectable drug.

139 *M. bovis* BCG Pasteur strain was cultured statically at 37°C, 5% CO<sub>2</sub> in liquid media (Middlebrook 7H9,  
140 Difco, supplemented with 10% (v/v) Middlebrook ADC, 0.05% (v/v) Tween-80 and 0.25% (v/v)  
141 glycerol) or solid media (Middlebrook 7H11 agar, Difco, with 0.5% (v/v) glycerol and 10% (v/v)  
142 Middlebrook OADC). The MIC of FNDR-20081 for *M. bovis* BCG, on solid media, was determined  
143 by plating out 10 µL spots of 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> and 10<sup>1</sup> cells onto 5 mL 7H11 agar plates of a series of 2-fold  
144 dilutions of the compound. The MIC was defined as the lowest concentration of FNDR-20081 that  
145 prevented any growth of the bacterium.

146 **Determination of minimum bactericidal concentration (MBC):** MBC was determined by a  
147 procedure reported previously [8]. Serial 2-fold dilutions of test molecule up to 6-fold of its MIC (0.25  
148 to 32 µg/mL) and INH (0.039-2.5 µg/mL) were prepared in DMSO and water respectively in a 96-well  
149 microtiter plate. A drug-free control was also included in the tests. All wells were inoculated with 200µl  
150 bacterial inoculum from the frozen enumerated stock of Mtb to give a final cell density of 1 ×10<sup>5</sup>  
151 CFU/mL in each well. The microtitre plate was incubated at 37°C for 7 days. MBC was determined by  
152 serial 10-fold dilution of these tubes using 7H9 broth or phosphate buffered saline (0.1 M, pH 7.4) as a  
153 diluent. Each dilution (0.5 mL) was plated in triplicate onto Middlebrook 7H10 agar supplemented with  
154 10% OADC and incubated at 37°C. The plates were counted for CFU on day 21 and day 28 of  
155 incubation. MBC was taken as the lowest concentration that killed 99% of the initial Mtb inoculum [11].

156 **Drug Combination MIC assay:** The *in vitro* drug interaction study was performed as described  
157 previously [12]. Briefly, synergistic/additive/antagonist interactions of test molecule with known anti-  
158 TB drugs against *M. tuberculosis* H37Rv (INH, RIF, EMB, AMK, CAP, STR, OFX and MXF, as well  
159 as the new drugs in the pipeline: Bedaquiline BDQ, Pretomanid/PA-824, and Linezolid LZD), were

160 evaluated by determining the MICs of the test molecule, anti-TB drugs alone and in combinations in 96-  
161 well plates by checkerboard method. Each combination was prepared so the mid-point concentration of  
162 each molecule equaled its MIC to capture synergism as well as antagonism if any (i.e., both the drugs  
163 were centering at their MIC). Serial dilutions were made in subsequent wells. Mtb culture was added as  
164 200µl in each well to give a bacterial density of approximately  $3-8 \times 10^5$  CFU/mL in each well. The plates  
165 were packed and incubated at 37°C for the next 6 days. The dye (Resazurin) was added on 6<sup>th</sup> day and  
166 the incubation was continued; the results were read by visual colorimetric inspection. MICs of each drug  
167 alone and in combination were described where the lowest concentrations showing no visible color  
168 change from blue to pink (i.e., no growth of *M. tuberculosis*) were considered minimum inhibitory  
169 concentrations. The combinatorial reductions in MICs were used to calculate the fractional inhibitory  
170 concentration (FIC). Fractional inhibitory concentration indices (FICI) were interpreted as follows:  $\leq 0.5$ ,  
171 synergism;  $>0.5-4.0$ , additive or indifference; and  $>4.0$ , antagonism.

172 **MIC determination in the presence of serum/albumin to determine protein binding:** MIC  
173 determination in the presence of serum/albumin was performed to evaluate the effect of protein binding,  
174 if any. This assay was performed by using a previously described broth microdilution assay, with minor  
175 modifications [13]. The MICs against *M. tuberculosis* H37Rv were determined under three different  
176 conditions: 1). without protein enrichment, 2). in the presence of 10% bovine serum albumin (BSA),  
177 and 3). in 10% fetal bovine serum (FBS).

178 **Mycobactericidal activity of FNDR-20081 on replicating Mtb to determine killing kinetics:** The  
179 killing kinetics assay on replicating Mtb population was performed as described previously [13,14,15].  
180 The Mtb (H37Rv) culture was inoculated at  $\sim 3-8 \times 10^7$  cfu/mL in fresh Middlebrook 7H9 complete  
181 medium containing varying concentrations of FNDR-20081 (0.015-256 µg/mL). The cultures were  
182 incubated at 37°C for different time points and enumerated, respectively. For the CFU enumeration,  
183 aliquots from the cultures containing different concentrations of the compounds were collected at day-  
184 3, day-7 and day-14 and plated at various dilutions ( $10^{-1}$  to  $10^{-8}$ ) to get countable colonies. Rifampicin  
185 was used as the assay quality control. Data was analysed and plotted as  $\log_{10}$  cfu/mL at day-3, day-7,  
186 and day-14 as a function of concentration of FNDR-20081 to calculate the range of concentration that  
187 shows killing potential.

188 **Mycobactericidal activity of FNDR-20081 on non-replicating Mtb:** FNDR-20081 was screened  
189 against non-replicating Mtb in three different models of the various simulated conditions [11,16], to test



190 its ability to kill various dormant populations of Mtb under: 1) low pH, 2) nutritional starvation and 3)  
191 stationary phase conditions. 1) Low pH model: Mtb was adapted to low pH condition by allowing it to  
192 grow under a pH of  $6.0 \pm 0.1$  at  $37^\circ\text{C}$  for 4 weeks in the Middlebrook 7H9 complete medium containing  
193  $\text{KH}_2\text{PO}_4$ , casein hydrolysate, glycerol. Low pH adapted Mtb inoculum was sub-cultured into previously  
194 described freshly made low pH media containing various concentrations (256 to  $0.5\mu\text{g/mL}$ ) of the  
195 FNDR-20081. PZA was used as a positive control for this assay. 2) Nutrient starvation model: the Mtb  
196 culture was pelleted, washed 2 times with phosphate-buffered saline (PBS) and reconstituted in PBS to  
197 remove all the nutrients from the media. This was followed by incubation at  $37^\circ\text{C}$  for 6 weeks to starve  
198 or nutritionally deprive the culture of Mtb. Starved cultures were exposed to various concentrations (256  
199 to  $0.5\mu\text{g/mL}$ ) of the compound. RIF was used as a positive control in this assay. 3) Stationary phase  
200 model: Mtb culture inoculated in Middlebrook 7H9 complete medium was incubated at  $37^\circ\text{C}$ / 6 months  
201 (to achieve stationary growth phase of Mtb) and was used for stationary phase model. A 6-month-old  
202 Mtb culture was diluted appropriately ( $\sim 10^7\text{cfu/mL}$ ) in the spent medium and exposed to various  
203 concentrations (1-256  $\mu\text{g/mL}$ ) of the compound. Rifampicin was used as a positive control for this assay.

204 The assay plates for the respective models were incubated at  $37^\circ\text{C}$  for 21 days. After 21-days, the  
205 respective exposed cultures were appropriately diluted ( $10^{-1}$  to  $10^{-8}$ ) and plated to get countable colonies  
206 as colony forming units (cfu) for enumeration of the survivors of compound exposed vs. the un-exposed  
207 controls. The data was analysed and plotted using GraphPad Prizm v5.0.

208 **Cytotoxicity:** Cytotoxicity of the compound was tested on HepG2, and phorbol 12-myristate 13-acetate  
209 (PMA) -activated THP-1 macrophage cell lines [12]. The compound was added at 2-fold concentrations  
210 ( $64$ - $0.125\mu\text{g/mL}$ ) to the respective cell lines. The plates were incubated at  $37^\circ\text{C}/5\% \text{CO}_2$  for 48 hrs. The  
211 colorimetric readings were taken after the addition of resazurin dye [12].

212 **Intracellular efficacy of FNDR-20081:** To test drug efficacy against slow or non-replicating bacilli in  
213 the intracellular compartment, tumor macrophage-derived cell line THP-1 was used. The THP-1 cells  
214 were grown in RPMI medium (Gibco-BRL Life Technologies, Gaithersburg, Md.) in  $75\text{-cm}^2$  flasks  
215 (*Corning Costar Corp., Cambridge, Mass.*).

216 RPMI complete media (100 mM sodium pyruvate, 200 mM L-glutamine, 3.7 g of sodium bicarbonate  
217 per liter (*SIGMA*), and 10% fetal bovine serum (*Gibco-BRL Life Technologies*)) was used without any  
218 antibiotics. The macrophages were counted in a hemocytometer, viability was determined by trypan

219 blue exclusion, and the macrophages were seeded in 24-well plates (Nunc, Roskilde, Denmark) with  
220 complete RPMI at a density of approximately  $5 \times 10^5$  cells/well and incubated overnight. The THP-1  
221 cells were differentiated by 50nM phorbol 12-myristate 13-acetate (PMA) induction to achieve  
222 macrophage phenotypes and were incubated at 37°C/48-72h/5% CO<sub>2</sub>. After 48 h of activation, the THP-  
223 1 macrophages were infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 1:10  
224 [12] and were incubated for 2 h at 37°C / 5% CO<sub>2</sub>. The medium containing the mycobacteria was  
225 discarded, macrophage monolayers were washed twice with 3 mL of PBS (+Ca<sup>2+</sup> + Mg<sup>2+</sup>) to remove the  
226 free bacteria and replenished with fresh complete RPMI. Sets of triplicate wells were lysed (0.05% SDS)  
227 at specific time-points and enumerated to estimate the numbers of intracellular Mtb 2hr post-infection  
228 [13,14,15]. The phosphate-buffered saline washed monolayers were lysed by adding 1 mL of water plus  
229 0.05% sodium dodecyl sulfate (SDS) for 5 min. The lysate was serially diluted and plated onto  
230 Middlebrook 7H11 agar plates (Difco Labs) for cfu enumeration (read after 3 to 4 weeks).

231 For the remaining wells, at 2 h post-infection the test compound (FNDR-20081) was added to sets of  
232 triplicate wells at respective concentrations (64-4-1 µg/mL) as well as the assay control RIF (16-4-1  
233 µg/mL). The final concentration of DMSO in the medium was maintained at 1% for all conditions. Sets  
234 of replicates from the infection control, test and the reference wells with each drug concentrations were  
235 sampled on 0, 3, 5, and 7 days. The wells were washed to remove the extracellular bacteria, if any,  
236 released after lysis of macrophages. The cell lysates were serially diluted and plated onto Middlebrook  
237 7H11 agar plates to estimate the numbers of intracellular viable mycobacteria. The intracellular  
238 mycobacterial killing rates of rifampin were generated by plotting the log<sub>10</sub> cfu/mL against the  
239 broth/MIC ratio and the AUC/MIC ratio.

240 **FNDR-20081 activity under different media conditions:** The in vitro growth inhibition of FNDR-  
241 20081 was tested on Mtb H37Rv growing under different media conditions, as per CLSI with  
242 modifications in media supplements respectively to mimic the protein-rich and lipid-rich host  
243 conditions. The activity was monitored in the presence of casitone, BSA, cholesterol, tyloxapol and Di-  
244 palmitoyl-phosphatidyl-choline (DPPC). The respectively adapted Mtb cultures were exposed to  
245 different concentrations of 2-fold diluted compound (256-0.5 µg/mL). The assay plates were incubated  
246 at 37°C for 14-days. The results were noted by visual turbidity.

247 **Activity against ESKAPE pathogens:** FNDR-20081 was tested against a panel of ESKAPE organisms  
248 to test its activity against Gram-positive and Gram-negative pathogens. The assay plates were incubated

249 for 24 hrs at 37°C. At the end of the assay visual turbidometric readings were taken and the results were  
250 noted [7,9,12].

251 **Generation of *Mycobacterium bovis* (*M. bovis*) BCG Spontaneous Resistance Mutants to FNDR-**  
252 **20081:** Target identification studies were performed through spontaneous mutant generation and whole  
253 genome sequencing (WGS). Over-expression studies were also used to confirm new targets identified  
254 by WGS of spontaneous resistant mutants.

255 To generate spontaneous mutants resistant to FNDR-20081, 10<sup>8</sup> cfu of log phase cells (OD<sub>600</sub> of 0.8-1.0)  
256 were plated out onto 7H11 agar containing 5×, 10× and 20× MIC of the compound. To confirm  
257 resistance, putative mutants were grown in liquid 7H9 media, in the absence of the compound, to log  
258 phase. The cells were spotted as 10 µL of 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> and 10<sup>1</sup> cfu on to 7H11 agar containing 5× MIC  
259 of FNDR-20081. Resistant mutants that grew on this plate were subjected to whole-genome sequencing  
260 (WGS) to determine mutations conferring resistance. Cells were grown to log phase in 50 mL liquid  
261 media, containing 3× MIC of FNDR-20081, and the genome purified. WGS and the alignment to the  
262 reference genome of *M. bovis* BCG Pasteur 1173P2 (accession number: NC\_008768.1) was completed  
263 by MicrobesNG.

264 **Target Gene Over-Expression:** Target genes were cloned into the plasmid pMV261, under the control  
265 of a constitutive promoter, in the presence of KAN (50 µg/mL) as selection marker. Compounds were  
266 serially diluted 2-fold, to obtain a range covering the MIC at 50× the desired concentration, and 2 µl  
267 was transferred across to 96-well Greiner black bottomed plates, leaving a single row moisture barrier  
268 around the outside of the plate. BCG or Mtb strains, containing the pMV261 plasmids, were grown to  
269 mid-log and diluted to 1.5 × 10<sup>6</sup> colony forming units (CFU)/mL. 98 µl of cells per well, was added to  
270 the assay plates, which were sealed and incubated at 37°C in a CO<sub>2</sub> incubator. After 7 days, 30 µl of a  
271 0.02% (w/v) solution of resazurin and 12.5 µl of 20% tween-80 was added and the plates were incubated  
272 overnight before measuring fluorescence (excitation at 530 nm and emission at 590 nm) using a  
273 POLARstar Omega plate reader (BMG Labtech.). MICs of target gene over-expressing strains were  
274 compared with a strain carrying a pMV261 empty vector. This method was also used to compare the  
275 liquid MICs of the spontaneous mutants generated to those of the wild type (WT) strain of BCG.

276 **Drug Metabolism and Pharmacokinetics of FNDR-20081:**

277 **Microsomal stability:** Microsomal stability was performed using human and mouse liver microsomes.  
278 The final composition of the assay included 1  $\mu$ M of FNDR-20081 and the final concentration of DMSO  
279 was 0.1%, 0.125 mg/mL microsomal protein and cofactors (5.0 mM G-6-P, 0.06 U/mL G-6-PDH, 2.0  
280 mM  $MgCl_2$ , 1.0 mM  $NADP^+$ ). The compound was incubated with human and mouse liver microsomes  
281 with and without cofactors. The reaction mixture was removed at specified time points (0, 15, 30, 60  
282 and 120 min) and the reaction was stopped by addition of ice-cold acetonitrile. The samples were  
283 extracted in presence of internal standard (Haloperidol) and were analyzed using LC-MS/MS. After the  
284 specified incubation period, percent of the remaining test/control compound was calculated with respect  
285 to the peak area ratio at time 0 min [7].

286 **CYP3A4 inhibition:** *In vitro* CYP3A4 enzyme inhibition assay was performed using human liver  
287 microsomes by probe substrate method. Serial dilutions of FNDR-20081 were prepared in potassium  
288 phosphate buffer (50 mM, pH 7.40) to obtain eight concentrations starting from 25  $\mu$ M. The final  
289 composition of the assay mix was acetonitrile 1%, microsomal protein as 0.25 mg/mL, probe substrate  
290 (midazolam, 5  $\mu$ M) and cofactors (5.0 mM G-6-P, 0.06 U/mL G-6-PDH, 2.0 mM of  $MgCl_2$ , 1.0 mM  
291  $NADP^+$ ). Serially diluted compound solutions and human liver microsomes were incubated for 10 min  
292 at 37°C with shaking. After pre-incubation, potassium phosphate buffer, probe substrate working  
293 solution and the cofactor mix was added. The reaction mixture was further incubated at 37°C with  
294 shaking (400 rpm) for 10 minutes. After incubation, reaction mixture was transferred to tubes containing  
295 stop solution (ice cold acetonitrile) and internal standard solution (Haloperidol), was centrifuged at  
296 10000 rpm for 10 minutes at 4°C. The supernatant was transferred to vials and submitted for LC-MS/MS  
297 analysis of marker metabolite (OH-Midazolam). The % CYP Inhibition at different tested concentrations  
298 relative to vehicle control was calculated and  $IC_{50}$  value was determined.

299 **Animals:** The *in-vivo* studies for FNDR-20081 were carried out in strict accordance with  
300 recommendations of the Institutional Animal Ethics Committee (IAEC), registered with the Committee  
301 for the Purpose of Control and Supervision (CPCSEA), Government of India (registration no.  
302 48/GO/Re-SL/BiS/99/CPCSEA). All the experimental protocols involving use of animals were  
303 reviewed and approved in advance by the IAEC. Carbon dioxide ( $CO_2$ ) was used for euthanasia. The  
304 BALB/c mice aged between 6 to 8 weeks with an average body weight of 20-30 grams were used.  
305 Animals were housed in Individually ventilated cages (IVC's) in BSL-3 conditions. Animals were

306 randomly assigned to cages and allowed to acclimatize for 1 week prior to experiments. Feed and water  
307 were provided *ad libitum*.

308 **Oral pharmacokinetics of FNDR-20081:** Single dose oral pharmacokinetic was established by  
309 administering the compound (at 30 mg/kg and 300 mg/kg) in fasted adult male BALB/c mice (about 8-  
310 10 weeks) formulated as suspension (in 5% (v/v) N,N-Dimethylacetamide (DMA), 5% Tween-80, 5%  
311 propylene glycol and 85% sterile water for injection) by oral gavage. Blood samples were withdrawn at  
312 specified intervals (Pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hours) into potassium –EDTA pre-coated  
313 tubes. Blood was centrifuged at 13000 rpm for 10 mins, plasma was separated and analysed using LC-  
314 MS/MS. Plasma samples were analyzed in LC-MS/MS and data was compiled by using non-  
315 compartment analysis using Win-Nonlin [7].

316 **Pharmacokinetics (PK) in infected animals:** PK was performed on Day-26 post infection. Infected  
317 animals were dosed once daily (30 mg/kg and 100 mg/kg). Blood samples were collected at different  
318 time intervals (1, 2, 4, 8 and 24 h, post dosing). Blood was centrifuged, plasma was separated and  
319 analysed using LC-MS/MS. Data was analysed by using non-compartment analysis using Winonlin.

#### 320 ***In-vivo* Efficacy of FNDR-20081:**

321 **Dose response in chronic infection model.** BALB/c mice were infected via aerosol inhalation in a  
322 Madison chamber calibrated to deliver 100 CFU/mouse lung [13]. Infected mice were housed in  
323 isolators (Allentown technologies, USA) during the entire period of experimentation. Treatment began  
324 4 weeks post infection. FNDR-20081 was administered once daily, by oral gavage in a vehicle (5% (v/v)  
325 DMA + 5% (v/v) Tween 80 + 5% (v/v) propylene glycol + 85% (v/v) sterile water). Three doses, 10,  
326 30 and 100mg/kg body weight were given 7 days a week, for a period of 4 weeks. RIF was used as a  
327 positive control. On completion of dosing, animals were sacrificed 48 hours later by CO<sub>2</sub> narcosis, lungs  
328 were removed, homogenized, and plated for enumeration of CFU/lung on Middlebrook 7H11 media  
329 plates supplemented with OADC and PANTA (BD-245114). The plates were incubated at 37°C with  
330 5% CO<sub>2</sub> for 3 weeks prior to reading bacterial CFU counts.

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## 334 Results

335 **Chemistry- Lead identified as FNDR-20081:** A library of 60 compounds was synthesized. The  
336 synthesis and preliminary SAR were reported earlier [7]. FNDR-20081 [4-{4-[5-(4-Isopropyl-phenyl)-  
337 [1,2,4]oxadiazol-3-ylmethyl]-piperazin-1-yl}-7-pyridin-3-yl-quinoline] was selected (MIC 0.5-  
338 2µg/mL) out of the quinoline series (Figure 1). FNDR-20081 series of compounds are novel and also  
339 amenable to SAR with handles to modulate drug like properties. Its synthetic route is less than 7 steps  
340 and therefore straightforward to scale up to multi gram production [7]. It is chemically stable, has no  
341 obvious toxicophoric properties and no reactive functional groups. FNDR-20081 was identified as the  
342 lead molecule for further progression.

343 FNDR-20081 is a small molecule with quinoline as a core (Figure 1). The quinoline has piperazine at  
344 the C-4-position and a 3 pyridyl at C-7. The distal nitrogen on piperazine is further coupled to 1,2,4-  
345 oxadiazole, which has a 4-isopropylphenyl at C-5. This is a unique scaffold with pyridine, quinoline,  
346 piperazine and 1,2,4-oxadiazole present in tandem. The synthesis and detailed SAR, along with the <sup>1</sup>H  
347 and <sup>13</sup>C NMR spectra was recently published [7]. FNDR-20081 was the most potent compound, hence  
348 was selected for further progression.

349 **Minimum inhibitory concentration and bactericidal activity of FNDR-20081 on Mtb H37Rv**  
350 **strain:** The compound demonstrated minimal inhibitory concentration (MIC) of 0.25-2µg/mL  
351 (0.5µg/mL) and the minimal bactericidal concentration (MBC) as just 2-fold of MIC (i.e., 4µg/mL)  
352 against *M. tuberculosis* H37Rv.

353 **Minimum inhibitory concentration of FNDR-20081 on drug resistant and sensitive Mtb strains:**  
354 FNDR-20081 is a promising compound with potent MICs against clinical isolates of Mtb of variable  
355 resistance profiles, with activity against sensitive, MDR and XDR strains. The MIC values of FNDR-  
356 20081 against a total of 61 Mtb strains with a wide spectrum of drug sensitivity [XDR(5), MDR(33),  
357 SDR (15), drug sensitive (8)] demonstrated very encouraging results. The MIC distribution was grouped  
358 into two categories: Low MIC range ( $\leq 4\mu\text{g/mL}$ ) and High MIC range (up to  $16\mu\text{g/mL}$ ) (Figure 2). The  
359 MIC values were within 4-fold range as compared to the WT Mtb strain ( $\leq 4\mu\text{g/mL}$ ) for all the XDR  
360 strains, 91 % of MDR, 80 % of SDR and 87 % of the sensitive strains of MIC of FNDR-20081. A total  
361 of 92% strains belonging to different resistance patterns fell under the category of Low MIC range  
362 ( $\leq 4\mu\text{g/mL}$ ), with MICs in the range of 1-16 µg/mL. None of the strains showed an MIC  $>16\mu\text{g/mL}$ .

363 FNDR-20081 retained activity against clinical isolates that included susceptible, INH-resistant, EMB-  
364 INH resistant, RIF-resistant and the other MDR/XDR isolates of Mtb.

365 **Drug Combination MIC assay:** The combination MIC of FNDR-20081 by checkerboard method  
366 showed no antagonism with any of the first line, second line drugs or the new drugs tested (**Table 1**).  
367 Drugs CLO and EMB showed synergism while all others exhibited indifference.

368 **MIC determination in the presence of serum/albumin to determine effect of protein binding:** MICs  
369 of FNDR-20081 and the standard anti-TB drug, INH/RIF, in un-supplemented media, media  
370 supplemented with 10% FBS and 10% BSA were determined to explore its protein binding capacity. In  
371 the presence of 10% BSA, the MIC of FNDR-20081 changed marginally by 2-fold (2 µg/mL) vs. un-  
372 supplemented media (1 µg/mL). The MICs of FNDR-20081 in the presence of 10% FBS and 10% BSA  
373 (a physiologically equivalent concentration of albumin) were negligible, being only 2-fold higher, than  
374 those in standard media. The MICs of INH and RIF also increased by 2- and 4-fold, respectively, under  
375 these supplementations. Thus, the protein binding percentage capacity of FNDR-20081 was comparable  
376 to that of reference anti-TB drugs.

377 The MIC of FNDR-20081 did not increase significantly when tested in protein rich (FCS and BSA) vs.  
378 the non-protein rich media. The MIC was changed only by 2-fold (2µg/mL) in FCS and BSA containing  
379 media (**Table 2**).

380 **Mycobactericidal activity of FNDR-20081 on replicating Mtb:** Bacterial colony forming units (cfu)  
381 were enumerated, data was compiled, and the kill curve graphs were generated (Figure 3) by plotting  
382  $\text{Log}_{10}$  CFU/mL values against time (h) in GraphPad Prizm v5.0.

383 *In-vitro* killing kinetics studies play an important role in determining the PKPD drivers for antibacterial  
384 activity and measuring maximum efficacy ( $E_{\text{max}}$ ) at various drug exposures. We have previously profiled  
385 PKPD parameters for anti-TB drugs and determined the PD driver for efficacy [13,14,15]  
386 recommending it for all the new compounds for their effective usage and suppression of drug resistance  
387 [13]. To investigate the killing kinetics of FNDR-20081 for determining the PD driver, 14-day kill-  
388 kinetics studies were performed using serial 4-fold dilutions (256-0.5µg/mL) against *M. tuberculosis*  
389 H37Rv. The kinetic kill-curve was generated by plotting  $\text{log}_{10}$  CFU vs. time at all concentrations (Figure  
390 3). FNDR-20081 displayed bactericidal effects with an  $E_{\text{max}}$  of 2.1  $\text{log}_{10}$  cfu/mL (Figure 3).

391 FNDR-20081 demonstrates increased kill with increasing concentrations (concentration-dependent) as  
392 well as after longer durations of exposures to the compound (time-dependent). The pattern of killing is  
393 very similar to the standard anti-TB drug RIF (and the superior drug Bedaquiline which is not compared  
394 in these models), which is one of the strongest known bactericidal anti-TB drugs under replicating as  
395 well as the non-replicating (NRP) conditions; reflecting the bactericidal potential of FNDR-20081 for  
396 the optimal treatment in clinical situations since Mtb exists under multiple replicating and non-  
397 replicating phases.

398 **Mycobactericidal activity of FNDR-20081 on non-replicating Mtb:** FNDR-20081 demonstrated a  
399 reasonable activity under low pH conditions, the most important intra-granuloma milieu for Mtb to  
400 survive and propagate [17]; reducing the Mtb load by 1.2 log<sub>10</sub> cfu/mL, FNDR-20081 also reduced the  
401 population of as well as on the nutritionally starved Mtb by 0.7 log<sub>10</sub> cfu/mL. However, under stationary  
402 phase it showed a negligible (0.2log<sub>10</sub> cfu/mL) cfu reduction (**Table 3**).

403 **Cytotoxicity of FNDR-20081:** Cytotoxicity of the compound was tested on HepG2 and PMA-activated  
404 THP-1 macrophage cell lines [12] at 2-fold diluted compound concentrations (64-0.125 µg/mL). The  
405 colorimetric readout confirmed that FNDR-20081 did not demonstrate any toxicity to THP-1 as well as  
406 HepG2 cells even up to 32X MIC (cytotoxicity >64 µg/mL).

407 **Intracellular efficacy of FNDR-20081:** The intracellular mycobacterial killing rates of FNDR-20081  
408 and the control drug rifampicin were determined [12] by nonlinear regression analysis (95% confidence  
409 limits). Inhibitory curves were generated by plotting the log<sub>10</sub> cfu/mL against the Day-0, 3, 5 and 7. The  
410 Emax observed was 1.5 log<sub>10</sub>cfu/mL (Figure 4)

411 **Activity of FNDR-20081 on Mtb in modified media/conditions:** FNDR-20081 was found to be active  
412 on Mtb growing under all the different nutrient source conditions, required for cell wall biosynthesis,  
413 hence, very unlikely to target cell wall biosynthesis (**Table 4**). This data suggests that FNDR-20081  
414 may be efficacious against Mtb *in-vivo* in the granuloma as well.

415 **Target identification of FNDR-20081:**

416 **Activity of FNDR-20081 on cell wall target over-expression in BCG:** Among the cell wall targets,  
417 initial studies to determine the mechanism of action of FNDR-20081 indicated that the compound does  
418 not target DprE1 (Figure 5). Over-expression of DprE1 in BCG shifts the MIC of the control compound



419 BTZ043 (known to target DprE1) by more than 16× (from 0.004 µg/mL to >0.064 µg/mL), when  
420 compared to cells with the pMV261 vector control. However, over-expression of DprE1 did not alter  
421 the MIC of FNDR-20081. In addition, there was no effect on the MIC of FNDR-20081 for resistant  
422 mutants generated to GSK303, another compound known to target DprE1 [18].

423 **FNDR-20081 Spontaneous Resistance Mutants in *M. bovis* BCG:** A systematic approach to target  
424 identification was adopted by the generation of spontaneous mutants. The MIC of FNDR-20081, for *M.*  
425 *bovis* BCG grown on solid media, was 6.25 µg/mL. Spontaneous resistant mutants grew at 5×, 10× and  
426 20× MIC of FNDR with frequencies of resistance (FoR) of  $7 \times 10^{-8}$ ,  $12 \times 10^{-8}$  and  $19 \times 10^{-8}$ , respectively.  
427 The genomes of four resistant mutants (one from 10× and three from 5× MIC) were sequenced and  
428 aligned to the genome of the parental strain to determine the mutations that could give rise to resistance.  
429 All four mutants carried mutations in the *marR* regulator (BCG\_0727, corresponding to Rv0678 in *Mtb*):  
430 a mutation in three of the mutants introduced a stop codon thereby interrupting gene expression; the  
431 other mutant had an insertion, resulting in a frame shift.

432 Since *MarR* regulates the expression of MmpL5, which is known to confer resistance to other drugs,  
433 such as azoles [19,20] through extrusion methods, cross-resistance of these *marR* mutants to an azole  
434 (clotrimazole, CLT) was compared to new spontaneous mutants generated to FNDR-20081 (Figure 6).  
435 The mutants with mutations in *marR* all demonstrated cross-resistance to CLT, with a 2-fold increase in  
436 MIC compared to the WT strain (25 vs. 12.5 µg/mL). However, there were three new mutants, resistant  
437 to FNDR-20081, with no cross-resistance to CLT. Mutations of all three mutants mapped to Rv3683, a  
438 metallophosphoesterase (Supplement Table S-1).

439 **Activity of FNDR-20081 against ESKAPE pathogens:** FNDR-20081 demonstrated a highly TB-  
440 specific activity. There was no MIC against any of the ESKAPE pathogens panel tested (**Table -5**).

441 **Drug Metabolism and Pharmacokinetics:**

442 **Microsomal stability and CYP3A4 inhibition:** The percentage of the compound remaining in the  
443 reaction mixture after specified incubation period was calculated with respect to the peak area ratio at  
444 time 0 min. FNDR-20081, was actively metabolized in mouse liver microsomes (<5% remaining at 60  
445 min), however it was moderately stable in human liver microsomes (40% remaining at 60 min) [7].

446 In addition, the CYP3A4 inhibition assay with FNDR-20081 revealed no CYP3A4 liability and the IC<sub>50</sub>  
447 was >25 μM. Other compounds in the series demonstrated CYP3A4 inhibition at <2μM.

448 **Oral pharmacokinetics of FNDR-20081:** Mean plasma concentrations of FNDR-20081 at 30 mg/kg  
449 and 300 mg/kg doses are shown in Figure 7. Orally bioavailable, a saturable absorption was observed  
450 for the compound (C<sub>max</sub>) of 7.4 μg/mL for the 30 mg/kg dose and 13.8 μg/mL for the 300 mg/kg dose  
451 group. There was a dose proportional increase in plasma exposures. AUC<sub>inf</sub> for the 30 mg/kg dose was  
452 11.9 h\*μg/mL and for the 300 mg/kg bw was 103.775 h\*μg/mL.

#### 453 **Pharmacokinetics in infected animals:**

454 The mean plasma concentration of FNDR-20081 administered at 30 mg/kg and 100 mg/kg bw p.o. doses  
455 were plotted (Figure 8). A 30mg/kg dose resulted in a C<sub>max</sub> of 3.2μg/mL which increased proportionally  
456 to 14.2μg/mL for the 100mg/kg dose; this which was equivalent to the 300 mg/kg dose in uninfected  
457 animals. The C<sub>max</sub> remained several folds above the MIC following repeat dosing. Similarly, there was  
458 increase in AUC<sub>last</sub> following repeat dosing of the compound (Figure 8A). A significant increase in MRT  
459 was observed from the 30mg/kg dose (4.7 h) to the 100 mg/kg dose (7.67 h).

#### 460 ***In-vivo* Efficacy of FNDR-20081:**

461 Based on ADME studies, FNDR-20081 was progressed for testing the *in-vivo* efficacy in the chronic  
462 mouse infection model of tuberculosis. Oral doses of 10, 30 and 100 mg/kg were tested. Treatment with  
463 FNDR-20081, was well tolerated in mice. There was no reduction in lung loads at 10 and 30 mg/kg  
464 doses but treatment with 100 mg/kg resulted in 0.6 log<sub>10</sub> CFU/lung (significant, p<0.05) reduction in  
465 bacterial counts (Figure 9). PK/PD correlations showed that PK parameters increased significantly for  
466 the 100mg/kg bw dose (C<sub>max</sub>: 14.2μg/ml and plasma AUC<sub>last</sub> 69.5 h\*μg/ml) and resulted in *in-vivo*  
467 efficacy. This suggested that efficacy is driven by PK parameters. RIF (30 mg/kg) used as a positive  
468 control drug reduced the bacterial loads by 3 log<sub>10</sub> CFU/lung.

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## 473 Discussion

474 Anti-microbial resistance (AMR) has posed major challenges in the clinical management of infectious  
475 diseases and shaken the health-care infrastructure. Tuberculosis alone has ~550,000 cases of MDR and  
476 XDR-TB and the overall cases have not declined much in recent years [1]. Although there are multiple  
477 reasons that drive the emergence of MDR, a major factor is the non-availability of new drugs targeting  
478 novel mechanisms. There is a plethora of old drugs, their toxicity and long treatment duration invariably  
479 results in poor compliance that leads to drug resistance. We need safer drugs targeting new mechanisms  
480 to combat the MDR-TB threat. There is an urgent medical need to combat the TB pandemic by  
481 developing novel and safer anti-tubercular drugs targeting pan-TB: sensitive, (S) multiple drug resistant  
482 tuberculosis (MDR-TB) and extensively drug resistant cases of tuberculosis (XDR-TB).

483 We discovered a novel, first in class anti-TB compound FNDR-20081 and hypothesized that it may play  
484 a key role in the treatment of active and latent (non-replicating) forms of TB and enable TB-eradication  
485 goals. We sought to design and explore quinoline in combination with piperazine and oxadiazole  
486 moieties, which makes FNDR-20081 an exclusive TB-specific inhibitor. This compound seems to have  
487 a distinct mechanism of action, with potent pan-TB activity irrespective of their resistance profile (MDR,  
488 XDR). More than 90% of the clinical isolates of variable sensitivity profiles had MIC in the low range  
489 ( $\leq 4\mu\text{g/mL}$ ) indicating its target novelty. Though quinolines and oxadiazoles have previously  
490 independently been reported to be antibacterial [21], as well as antimycobacterial agents [22-35], their  
491 oral exposure suggested solubility limited drug absorption in addition to the first pass metabolism  
492 problems. Piperazine compounds [36-38] have also been reported as potent anti-TB agents against  
493 MDR-TB.

494 We could successfully generate spontaneous mutants to the compound. Initially, mutations disrupted the  
495 expression of *marR*, a Mar-like (multiple antibiotic resistance) transcriptional repressor Rv0678  
496 [20,39,40] responsible for the regulation of the *mmpS5-mmpL5* operon [19]. Mutations within this  
497 regulator have also been linked with resistance to azoles [19]. These mutations were shown to upregulate  
498 expression of *mmpS5-mmpL5* operon and coincided with a reduced level of azoles measured within the  
499 cytoplasm of the mutants, leading the authors to speculate that this region may encode an efflux pump  
500 [19] (*Milano et al., 2009*). Indirectly or directly MmpL5 along with MmpL4 has also been reported to  
501 block iron transport through mycobacterial siderophores (mycobactins and carboxymycobactins,  
502 [20,41,42]) leading to siderophore mediated suicidal intracellular pools. These Mtb mutants generated

503 against FNDR-20081 do demonstrate cross-resistance to CLT. But interestingly, in the other three  
504 subsequent spontaneous mutants to FNDR-20081, that had no mutations in *marR* and were not cross-  
505 resistant to CLT, mapped to BCG\_3742, corresponding to Rv3683 in Mtb. Rv3683 is a  
506 metallophosphoesterase, while non-essential by transposon site hybridization (TraSH) [43], the location  
507 of Rv3683 to a penicillin binding protein (PBP) on the genome could form part of a more significant  
508 interaction with this protein and peptidoglycan biosynthesis. Additional studies will be required to  
509 further validate Rv3683 also as a target of FNDR-20081.

510 Undoubtedly, MDR can be counteracted by targeting novel targets using combination of novel  
511 drugs/chemical entities. A recent study by Conradie et al. (2020) and Global Alliance for TB has  
512 unequivocally confirmed this hypothesis [4]. A combination of three recently developed new drugs  
513 Bedaquiline, Pretomanid and Linezolid (BPaL) reduced treatment of MDR-TB from usual 24 months to  
514 6 months [4]. This is the first evidence after 70 years that MDR-TB can be treated in shorter periods  
515 with the potency of new drugs targeting novel bacterial targets. This has brought in a new paradigm that  
516 MDR-TB may not be necessarily hard to treat, provided that we can hit new bactericidal targets with  
517 new chemical scaffolds.

518 Another emerging concept that could impact treatment of MDR-TB is multitarget therapy [4]. “Multi-  
519 targeting” therapy, with a single drug is epitomized to be a preferred approach over conventional mono-  
520 targeting [20,44-48]. Many of the successful anti-TB regimen drugs (e.g., isoniazid, ethambutol,  
521 pyrazinamide and Pretomanid) [48] as well as another drug in the pipeline, ethylene diamine drug,  
522 SQ109 (an uncoupler inhibiting two distinct proteins involved in cell wall and menaquinone  
523 biosynthesis (Mmp13, MenA and MenG, and ATP biosynthesis proteins) exhibit multi-targeting  
524 phenomenon [48-50]. Among the existing drugs, coumarins (e.g., Novobiocin), inhibit DNA  
525 topoisomerases along with Fad24 and FtsZ [49]. Such multi-targeting drugs are powerful tools to tackle  
526 multi-drug-resistance. FNDR-20081 also probably hits multiple targets within the cell wall such as  
527 regulator of *mmpS5-mmpL5* and a metallo-phosphoesterases. However, more intricate studies are  
528 required to confirm the targets with identification of the precise ligand binding site. It may provide an  
529 opportunity to develop new combinations against drug resistant Mtb.

530 The pathogen and the host have co-evolved with a strong association towards manipulating a fine  
531 balance in establishing the disease or no-disease; and dissemination or persistence [16,51]. Mtb  
532 encounters hostile conditions under both extracellular as well as intracellular milieu in the host and may

533 consequently adopt a replicating or a non-replicating phenotype [16,17,52]. One-quarter of the world's  
534 population is infected with a latent form of TB which is presumed to be non-replicating and  
535 metabolically inactive phenotype [1]. Non-replicating populations (NRP) are produced under *in-vitro*  
536 stress conditions (acidic, nutrient starvation, oxygen deprivation, stationary phase conditions as well as  
537 in macrophages) [8,16,17,52]. These non-replicating forms are unusually drug tolerant. Interestingly,  
538 FNDR-20081 is active against all the phenotypes of Mtb (Table 3). Multitargeting seems to be an  
539 emerging concept in TB drugs. All recently discovered anti-TB drugs (Bedaquiline, Pretomanid and  
540 Delamanid) hit multiple targets and are active against both replicating and non-replicating populations  
541 of Mtb [53,54]. FNDR-20081 is bactericidal, killed  $>2\log_{10}$  CFU of replicating Mtb in an exposure-  
542 dependent manner at day-14. However, the kill was not saturated suggesting that FNDR-20081 may  
543 indeed have even better bactericidal potential.

544 The ability of FNDR-20081 to kill nonreplicating Mtb (NRP-Mtb) under different physiological  
545 conditions along with the desirable PK properties showed translation into *in-vivo* efficacy in the chronic  
546 TB infection model harboring mixed populations of replicating and NRP-Mtb in the lungs of mice. This  
547 effect of FNDR-20081 on NRP forms in mice gives us optimism that it will likewise kill the  
548 dormant/persistent bacteria in latently infected human patients.

549 Metabolic studies in human and mouse microsomes revealed FNDR-20081 to be unstable (moderate in  
550 HLM and poor in MLM) suggesting the role of hepatic metabolism in the elimination of compound [7].  
551 Nevertheless, plasma Cmax levels were above the MIC with high plasma exposures for most of the  
552 doses that resulted in *in-vivo* efficacy.

553 The PK levels above the MIC translated into bacterial reduction by 0.56  $\log_{10}$  CFU/lung at a 100 mg/kg  
554 dose. A significant increase in AUC/MIC (8-fold) as well as time/MIC (1.7-fold) observed for the 100  
555 mg/kg bw dose may be the key factor driving efficacy as compared to the 10 mg/kg and 30 mg/kg  
556 dosing. The data further strengthened the *in-vitro* observation of exposure (concentration- as well as  
557 time)- driven efficacy in kill kinetics experiments. Based on proportionally higher PK exposure observed  
558 at 300 mg/kg, we firmly believe that the *in-vivo* efficacy can be improved further at higher doses.

559 FNDR-20081 is a highly TB-specific preclinical candidate that can be optimized further into a clinical  
560 candidate. An exclusive use of this molecule against MDR TB probably will give an opportunity for a  
561 restricted use to avoid an indiscriminate use leading to emergence of resistance.

562 To conclude, we report FNDR-20081, a novel first in class oral compound with a multitargeting  
563 mechanism of action, acting on a transcriptional repressor responsible for regulation of *mmpS5-mmpL5*  
564 operon and a metallo-phospho-esterase that needs to be intricately delineated further. FNDR-20081 is  
565 active against replicating and non-replicating populations of TB under *in-vitro*, intra-macrophage, and  
566 *in-vivo* conditions. In addition, compatibility for combination therapy and no drug-drug interaction of  
567 FNDR-20081 with existing TB drugs supports possibility of developing novel anti-TB drug regimens.

568 Therefore, next, we plan to test the *in-vivo* efficacy at the higher tolerable dose of 300mg/kg bw and in  
569 combination with the 1<sup>st</sup> line, 2<sup>nd</sup> line TB drugs, as well as with the new drugs recently entered in clinical  
570 use such as Bedaquiline, Pretomanid and Linezolid (BPaL). In parallel, further studies to investigate its  
571 potential to shorten duration of treatment time needs to be explored.

## 572 **Funding**

573 This study was funded by Foundation for Neglected Disease Research.

574

## 575 **Declaration of competing interest**

576 The authors declare no competing interests.

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586 **ACKNOWLEDGEMENTS**

587 The authors would like to acknowledge Mr. Mayas Singh from FNDR for facilitating the project  
588 activities.

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824 **Tables.**

825 **TABLE 1:** FNDR-20081: combination MIC with SoC 1st line, 2nd line and the new drugs in pipeline

Sl.no	2 drug combination		FIC index	Outcome
<b>Invitro combination studies with First line drugs</b>				
1	FNDR-20081	Isoniazid	1.29	Additive
2	FNDR-20081	Rifampicin	1.94	Indifference
3	FNDR-20081	Ethambutol	0.54	Synergy
<b>Invitro combination studies with Second line drugs</b>				
1	FNDR-20081	Capreomycin	2.99	Indifference
2	FNDR-20081	Kanamycin	2.99	Indifference
3	FNDR-20081	Streptomycin	3.09	Indifference
4	FNDR-20081	D-Cycloserine	1.70	Indifference
5	FNDR-20081	Amikacin	3.07	Indifference
6	FNDR-20081	Clofazimine	0.46	Synergy
7	FNDR-20081	Moxifloxacin	2.58	Indifference
<b>In-vitro combination studies with New TB drugs</b>				
1	FNDR-20081	Bedaquiline	1.06	Additive
2	FNDR-20081	PA-824	1.16	Additive
3	FNDR-20081	Linezolid	1.07	Additive
4	FNDR-20081	SQ109	1.94	Indifference

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828 **TABLE 2:** Serum/ Plasma protein binding.

MIC Mtb ( $\mu\text{g/ml}$ )	7H9	7H9+10%FCS	7H9+10%BSA
FNDR-20081	0.5-1	2	2
RIF	0.0125	0.05	0.025
INH	0.06	0.12	0.12

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832 **TABLE 3:** FNDR-20081 activity: **a.** under Low pH (1.2 log<sub>10</sub> cfu/mL), Nutrient starvation (0.7 log<sub>10</sub>  
 833 cfu/mL), stationary phase (0.2 log<sub>10</sub> cfu/mL), **b.** Kill against replicating vs. non-replicating Mtb

a.

Conc. µg/ml	Emax (Log <sub>10</sub> cfu reduction)					
	LpH		NSM		STA	
	20081	PZA	20081	RIF	20081	RIF
1	0.53	0.26	0.12	-0.06	-0.32	1.77
4	0.61	0.28	0.27	2.46	-0.16	2.25
16	0.75	0.55	0.51	2.95	-0.04	2.61
64	0.95	1.60	0.57	3.31	0.10	2.62
256	1.18	1.68	0.69	nd	0.18	nd

834 b.

Condition	Emax (Log <sub>10</sub> cfu reduction)			
	Day	20081-256	RIF-64	PZA-256
REP-KK	D-14	2.1	nd	nd
LpH	D-21	1.2	nd	1.7
NSM	D-21	0.7	3.3	nd
STA	D-21	0.2	2.6	nd

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836 **TABLE 4.** FNDR-20081: Activity against Mtb growing under a variety of nutritional sources

FNDR	1-week MIC 7H9/glucose/ casitone/Tx	2-week MIC 7H9/glucose/ casitone/Tx	1-week MIC 7H9/glucose/ BSA/Tx	2-week MIC 7H9/glucose/ BSA/Tx	1-week MIC 7H9/DPPC/ casitone/Tx	2-week MIC 7H9/DPPC/ casitone/Tx	1-week MIC 7H9/DPPC/ cholesterol/ BSA/Tx	2-week MIC 7H9/DPPC/ cholesterol/ BSA/Tx	Likely mycolyl- arabinogalactan biosynthetic inhibitor?
	ug/mL	ug/mL	ug/mL	ug/mL	ug/mL	ug/mL	ug/mL	ug/mL	
20081	6.1	12.2	12.2	18.06	9.27	9.27	12.2	18.06	No

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838 **TABLE 5.** FNDR-20081: Activity against ESKAPE pathogens: *Enterococcus faecium* [VRE],  
 839 *Staphylococcus aureus* [MRSA], *Klebsiella pneumoniae* [sensitive], *Acinetobacter baumannii*  
 840 [sensitive], *Pseudomonas aeruginosa* [sensitive], *Enterobacter aerogenes* [sensitive]

MICROBIOLOGY	MIC (µg/ml)					
Compound No.	<i>Enterococcus faecium</i>	<i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i>	<i>Acinetobacter baumannii</i>	<i>Pseudomonas aeruginosa</i>	<i>Enterobacter aerogenes</i>
Strains details	Vancomycin-R	Methicillin-R	Drug-sensitive	Drug-sensitive	Drug-sensitive	Drug-sensitive
FNDR-20081	>32	>32	>32	>32	>32	>32



841 **Figure Legends**

842 **Figure 1.** Structure of FNDR-20081. Oxadiazole-piperazine-quinoline in tandem (4-{4-[5-(4-  
843 Isopropyl-phenyl)-[1,2,4]oxadiazol-3-ylmethyl]-piperazin-1-yl}-7-pyridin-3-yl-quinoline). Mtb MIC  
844 0.5µg/ml (1.02µM).

845 **Figure 2.** MIC of FNDR-20081 against 61 Mtb strains of different resistance patterns [Blue bar=WT  
846 (1) Mtb strain, red bars=XDR(5), yellow bars=MDR(33), purple bars=SDR (15), and green bars=drug  
847 sensitive (8) Mtb strains] grouped under two categories: low MIC range ( $\leq 4\mu\text{g/mL}$ ) and high MIC range  
848 (up to  $16\mu\text{g/mL}$ ). Total  $\geq 92\%$  strains were sensitive to FNDR-20081. n= number of isolates under  
849 respective low MIC or high MIC categories.

850 **Figure 3.** Killing kinetics of FNDR-20081. Residual  $\log_{10}$  cfu/ml of Mtb post exposure to different  
851 concentrations of FNDR-20081 tested from 0.015 to  $256\mu\text{g/ml}$ , enumerated on day-0, day-3, day-7, and  
852 day-14. Time and concentration dependent (AUC) killing kinetics were demonstrated. FNDR-20081  
853 showed an  $E_{\text{max}}$  of **2.1**  $\log_{10}$  cfu/mL.

854 **Figure 4.** Efficacy of FNDR-20081 tested at different concentrations (0.03, 0.125, 0.5, 2 and  $8\mu\text{g/ml}$ )  
855 against intracellular Mtb in THP-1 macrophages on day-0, day-3, day-5, and day-7.  $E_{\text{max}}=1.5\log_{10}$   
856 cfu/mL

857 **Figure 5.** Activity of FNDR-20081 against DprE1 target over-expression in *M. bovis* BCG. A. Over-  
858 expression of DprE1 conferred a greater than 16-fold increase in resistance to BTZ043 (assay control),  
859 B. But no resistance to FNDR-20081.

860 **Figure 6.** Cross-resistance of FNDR-20081 spontaneous mutants to clotrimazole (CLT). MIC of the  
861 mutants generated to FNDR-20081 was examined against CLT and FNDR-20081. Live bacteria were  
862 identified by measuring fluorescence after resazurin addition (fluorescence of 100000 vs. 1000).

863 **Figure 7.** Drug metabolism and Pharmacokinetics (PK) of FNDR-20081. A. Drug metabolism. B.  
864 Tabulated PK parameters at 30 and 300 mg/kg bw. C. Single dose *in-vivo* PK at two different doses.  
865 Abbreviations used: MLM= Mouse Liver Microsomes, HLM= Human Liver Microsomes, MRT= Mean  
866 Retention time.

867 **Figure 8.** Pharmacokinetics of FNDR-20081 in infected mice. A. Tabulated PK parameters. B. PK  
868 profiles of 30 and 100 mg/kg doses.

869 **Figure 9.** *In-vivo* efficacy of FNDR-20081 at 10, 30 and 100mg/kg bw in chronic Mtb infection model  
870 in BALB/c mice. Dose of 100mg/kg bw demonstrated significant ( $P<0.05$ ) 0.56 log<sub>10</sub> CFU/lung  
871 reduction vs. untreated control.

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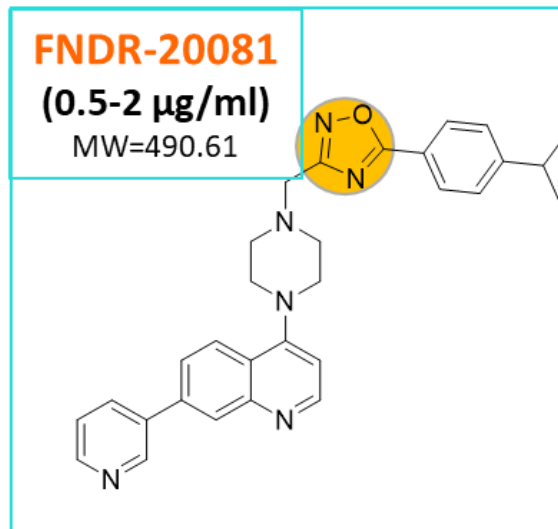
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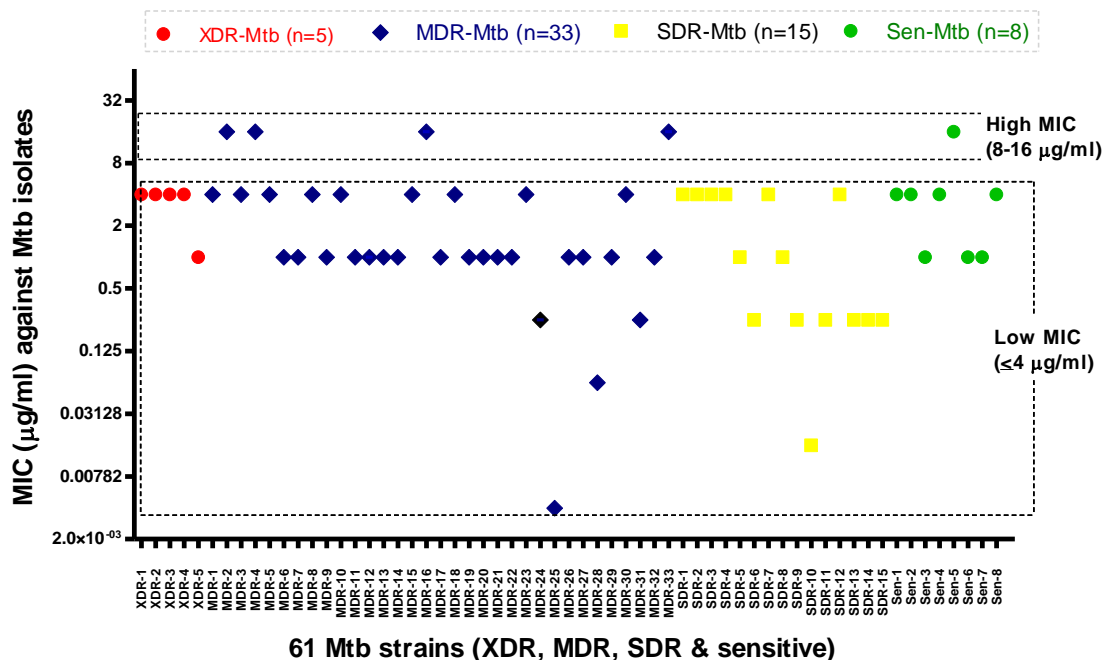
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890 **Figures:**



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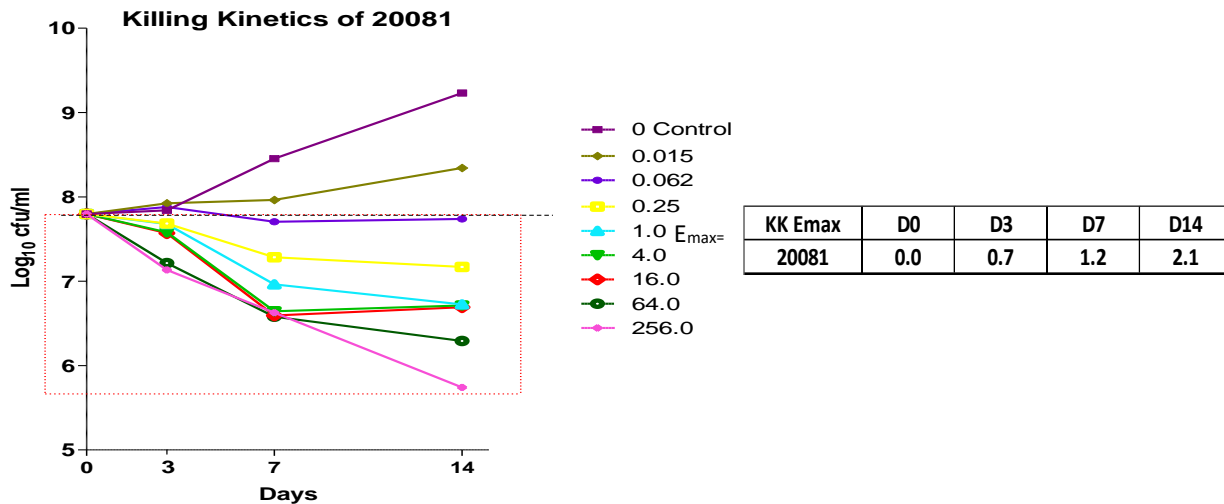
892 **FIG 1:** FNDR-20081. Oxadiazole--piperazine-quinoline in tandem (4-{4-[5-(4-Isopropyl-phenyl)-  
893 [1,2,4] oxadiazol-3-ylmethyl]-piperazin-1-yl}-7-pyridin-3-yl-quinoline). Mtb MIC 0.5 $\mu\text{g/ml}$  (1.02 $\mu\text{M}$ ).



894

895 **FIG 2.** Total 61 Mtb strains from different resistance patterns [XDR (5), MDR (33), SDR (15), drug  
896 sensitive (8)] grouped under into two categories: Low MIC range ( $\leq 4 \mu\text{g/ml}$ ) and High MIC range  
897 (up to  $16 \mu\text{g/ml}$ ). Total  $\geq 92\%$  strains were sensitive to FNDR-20081.

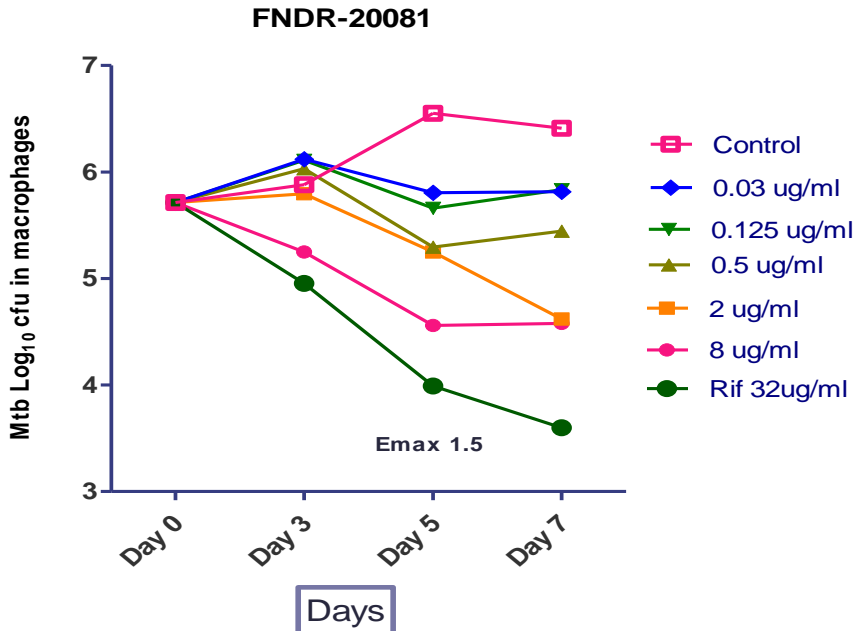
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900 **FIG 3.** Killing kinetics: FNDR-20081 exhibited time and concentration dependent (AUC) killing  
 901 kinetics, FNDR-20081 is a bactericidal compound,  $E_{max} = 2.1 \log_{10} \text{cfu/mL}$

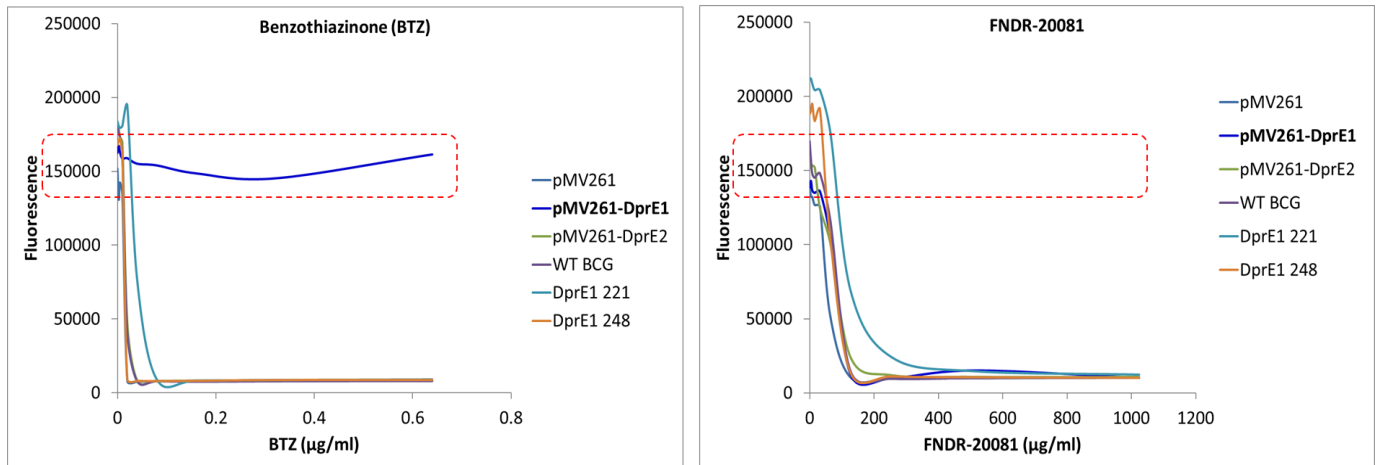
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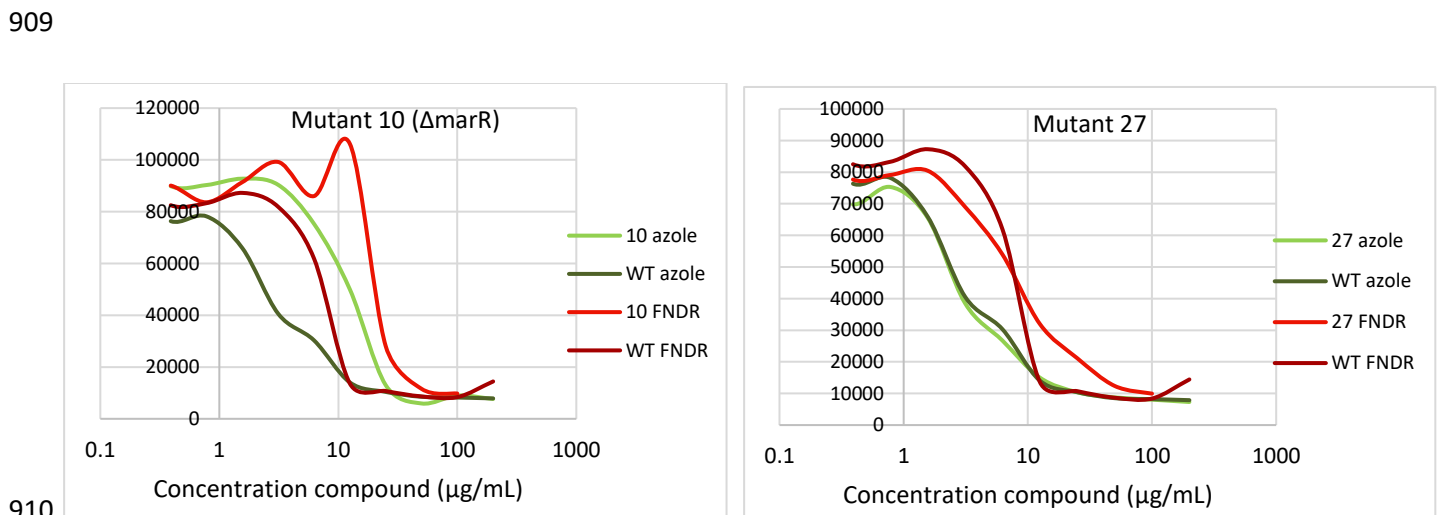
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904 **FIG 4.** Intracellular efficacy  $E_{max} = 1.5 \log_{10} \text{cfu/mL}$

905



906  
 907 **FIG 5.** Over-expression studies of DprE1 in *M. bovis* BCG. Over-expression of DprE1 confers a  
 908 greater than 16-fold increase in resistance to BTZ043, but no resistance to FNDR-20081.



910  
 911  
 912 **FIG 6.** Cross-resistance of FNDR-20081 spontaneous mutants to clotrimazole. The MIC of the mutants  
 913 generated to FNDR-20081 was examined in liquid culture, for clotrimazole and FNDR-20081, using a  
 914 2-fold serially diluted range of drugs in a 96 well plate. Live bacteria were identified by measuring  
 915 fluorescence after resazurin addition (fluorescence of 100,000 c.f. 1000).

916

917 a.

Parameter		FNDR-20081
Liver microsomal stability (%)	MLM	1.22
	HLM	40.1
CYP 3A4 inhibition	IC <sub>50</sub> (μM)	>25
HepG2 cell toxicity (%)	100 μM	38.7

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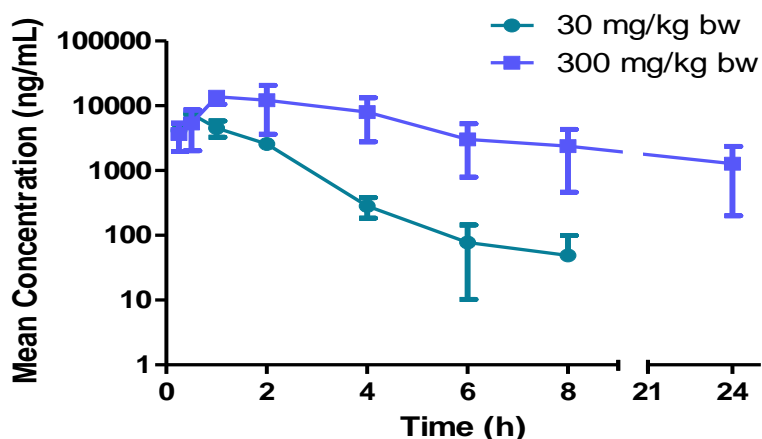
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924 b.

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Parameters	30 mg/kg, po	300 mg/kg po
C <sub>max</sub> (μg/mL)	7.4	13.8
T <sub>max</sub> (h)	0.5	1
AUC <sub>last</sub> (h*μg/mL)	11.9	85.3
AUC <sub>inf</sub> (h*μg/mL)	11.9	103.8
AUC <sub>extrap</sub> (%)	0.59	17.78
MRT <sub>last</sub> (h)	1.38	6.64

932 c.

933

934 **FIG 7.** Pharmacokinetics of FNDR-20081. Tabulated and plotted Single dose in-vivo pharmacokinetics  
935 at two different doses.

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937 a.

PK parameters of FNDR-20081 in infected animals (Day-26, repeat dosing)		
Parameters	30mg/kg	100mg/kg
$C_{max}$ ( $\mu\text{g/mL}$ )	3.2	14.2
$AUC_{last}$ ( $\text{h}\cdot\mu\text{g/mL}$ )	8.3	69.5
$MRT_{last}$ (h)	4.7	7.7
<i>In-vivo</i> efficacy (Chronic Model)	-0.2	0.6

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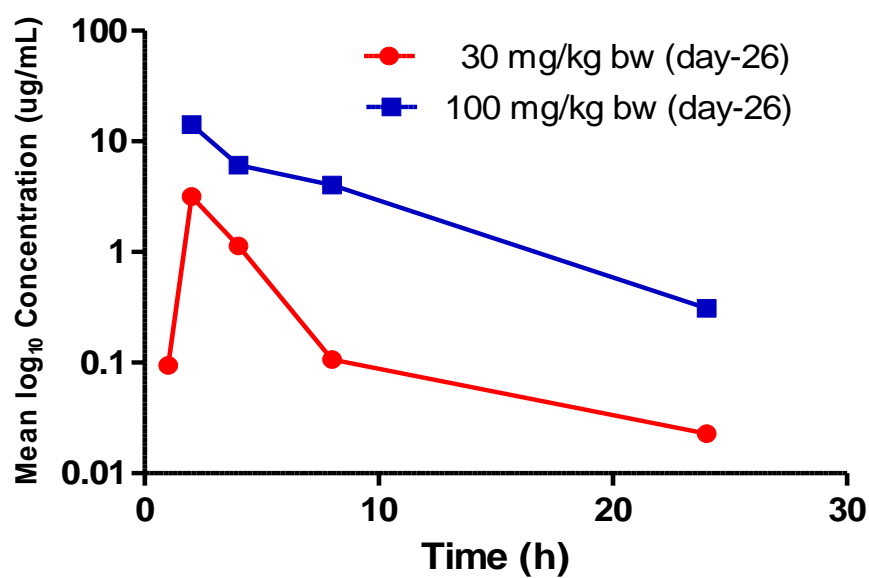
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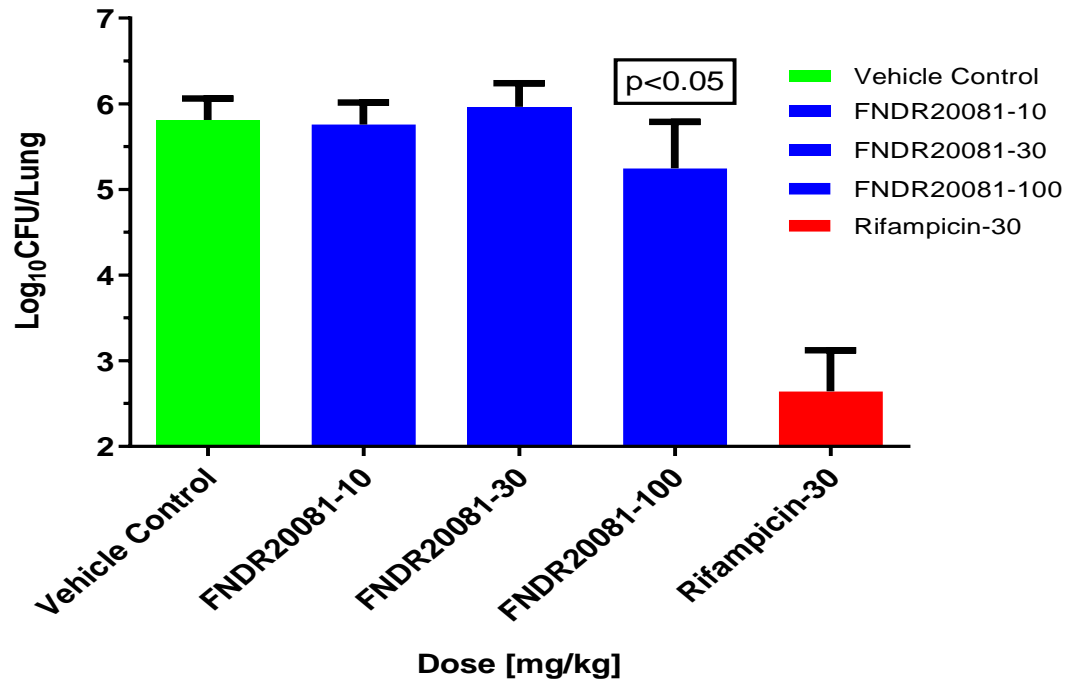
945 b.

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947 **FIG 8.** Pharmacokinetics of FNDR-20081 in infected mice. **a:** Tabulated values. **b:** Plotted day-26 in-  
948 vivo pharmacokinetics in infected animals at two different doses.

949

FNDR-20081: Efficacy in chronic infection model of TB



950

951 **FIG 9.** *In-vivo* efficacy of FNDR-20081 in BALB/c mice demonstrated significant ( $P < 0.05$ ) 0.56 log<sub>10</sub>  
952 CFU/lung reduction.

953



Table S1. FNDr-20081 spontaneous resistance mutants in BCG. WGS data and mutation mapping.

CHROM	POS	TYPE	REF	ALT	EVIDENCE	FTY	STR	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE	PRODUCT	FNDR22	FNDR23	FNDR27	number_of_pos_with_variant
NC_008769	1E+06 complex		CG	GC	GC:8 CG:0												1
NC_008769	2E+06 snp		G	C	C:5 G:0	CDS	-	1926/2223	642/740	synonymous_variant c.1926C>G p.Gly642Gly	BCG_1513c	Rv1759c	PE family protein	C:5 G:0	GC:8 CG:0		1
NC_008769	2E+06 complex		CG	CCGGC	CCGGC:7 CG:CDS	CDS	-	666/2690	222/895	disruptive_insertion_synonymous_variant c.666delCinsGGCC p.Trh222_Val223insPro	BCG_1799c	Rv1759c	hypothetical protein	CCGGC:7 CG:CCGGC:7 CG:CCGGC:6 CG:			non essential gene by Himar1-based transposon 3 mutagenesis in H37Rv strain (see Sasseti et al., 2003) deleted in some clinical isolates
NC_008769	3E+06 snp		T	A	A:36 T:0	CDS	-	531/3414	177/1137	synonymous_variant c.531A>T p.Trh177Thr	BCG_2507c		LuR family transcriptional regulator	A:36 T:0 A:42 T:0 A:33 T:0			3
NC_008769	3E+06 ins		TTA	TATA	TATA:36 TTA:CDS +	CDS	+	75/1743	25/580	frameshift_variant c.74_75insA p.Phe25fs	BCG_2963		long-chain-fatty-acid-AMP ligase FadD28	TATA:36 TTA:0			1
NC_008769	4E+06 snp		C	T	T:26 C:0	CDS	-	102/1158	34/385	synonymous_variant c.102G>A p.Pro34Pro	BCG_3265c		catonproton antiporter	T:26 C:0 T:46 C:0 T:31 C:0			3
NC_008769	4E+06 ins		GGC	GGCCGC	GGCCGC:17 CDS	CDS	-	1296/1419	432/472	conservative_insertion p.1296_1297insGGC p.Ala432dup	BCG_3499c	Rv3433c	bifunctional ADP-dependent NAD(P)H-hydrate dehydratase/NAD(P)H-hydrate epimerase	GGCCGC:17 GGCCGC:13 GGCC:0			non essential gene by Himar1-based transposon 2 mutagenesis in H37Rv strain (see Sasseti et al., 2003) disruption causes growth advantage in vitro
NC_008769	4E+06 ins		GGC	GGTCGC	GGTCGC:18 CDS	CDS	+	783/786	261/261	disruptive_insertion c.783_783insTCG p.Gly261_Ter262insAlaG	BCG_3517	Rv3451	cutinase family protein	GGTCGC:18 GGTCGC:21 C:GGTCGC:9 G:			3 KO is more resilient to stress
NC_008769	4E+06 snp		A	G	G:5 A:0	CDS	+	1795/4119	599/1372	missense_variant c.1795A>G p.Asn599Asp	BCG_3571		PE family protein	G:5 A:0 G:7 A:0 G:10 A:0			3
NC_008769	4E+06 snp		C	T	T:11 C:0	CDS	+	2115/4119	705/1372	synonymous_variant c.2115C>T p.Gly705Gly	BCG_3571		PE family protein	T:11 C:0 T:10 C:0 T:15 C:0			3
NC_008769	4E+06 snp		A	G	G:5 A:0	CDS	+	1792/3228	598/1075	missense_variant c.1792A>G p.Trh598Ala	BCG_3577		PE family protein	G:5 A:0 G:6 A:0			2
NC_008769	4E+06 snp		C	A	A:40 C:3	CDS	+	564/960	188/319	missense_variant c.564C>A p.Asp188Glu	BCG_3742	Rv3683	metallophosphoesterase	A:40 C:3 A:41 C:0 A:27 C:0			non essential gene by Himar1-based transposon 3 mutagenesis in H37Rv strain (see Sasseti et al., 2003)
NC_008769	4E+06 snp		C	G	G:34 C:3	CDS	-	1128/1530	376/509	missense_variant c.1128G>C p.Leu376Phe	BCG_3755c	gJpK	glycerol kinase GpK	G:34 C:3 G:35 C:0 G:23 C:0			3
NC_008769	705623 complex		GTGG	ATGC	ATGC:7 GTGCDS	CDS	-	1176/3312	391/1303	synonymous_variant c.1173_1176delCCACinsGCAT p.393	BCG_0623c		PE family protein	ATGC:7 GTGC:0			1