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A Multi-Targeting Pre-Clinical Candidate against Drug-Resistant Tuberculosis

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Abstract

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

Keywords

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

Introduction

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

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

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

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

Materials and methods

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

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

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

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

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

M. bovis BCG Pasteur strain was cultured statically at 37˚C, 5% CO_2 in liquid media (Middlebrook 7H9, Difco, supplemented with 10% (v/v) Middlebrook ADC, 0.05% (v/v) Tween-80 and 0.25% (v/v) glycerol) or solid media (Middlebrook 7H11 agar, Difco, with 0.5% (v/v) glycerol and 10% (v/v) Middlebrook OADC). The MIC of FNDR-20081 for *M. bovis* BCG, on solid media, was determined by plating out 10 µL spots of 10^4, 10^3, 10^2 and 10^1 cells onto 5 mL 7H11 agar plates of a series of 2-fold dilutions of the compound. The MIC was defined as the lowest concentration of FNDR-20081 that prevented any growth of the bacterium.

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

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

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

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

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

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

its ability to kill various dormant populations of Mtb under: 1) low pH, 2) nutritional starvation and 3) stationary phase conditions. 1) Low pH model: Mtb was adapted to low pH condition by allowing it to grow under a pH of 6.0±0.1 at 37°C for 4 weeks in the Middlebrook 7H9 complete medium containing KH_2PO_4, casein hydrolysate, glycerol. Low pH adapted Mtb inoculum was sub-cultured into previously described freshly made low pH media containing various concentrations (256 to 0.5µg/mL) of the FNDR-20081. PZA was used as a positive control for this assay. 2) Nutrient starvation model: the Mtb culture was pelleted, washed 2 times with phosphate-buffered saline (PBS) and reconstituted in PBS to remove all the nutrients from the media. This was followed by incubation at 37°C for 6 weeks to starve or nutritionally deprive the culture of Mtb. Starved cultures were exposed to various concentrations (256 to 0.5µg/mL) of the compound. RIF was used as a positive control in this assay. 3) Stationary phase model: Mtb culture inoculated in Middlebrook 7H9 complete medium was incubated at 37°C/ 6 months (to achieve stationary growth phase of Mtb) and was used for stationary phase model. A 6-month-old Mtb culture was diluted appropriately (~10^7cfu/mL) in the spent medium and exposed to various concentrations (1-256 µg/mL) of the compound. Rifampicin was used as a positive control for this assay.

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

Cytotoxicity: Cytotoxicity of the compound was tested on HepG2, and phorbol 12-myristate 13-acetate (PMA) -activated THP-1 macrophage cell lines [12]. The compound was added at 2-fold concentrations (64-0.125µg/mL) to the respective cell lines. The plates were incubated at 37°C/5% CO_2 for 48 hrs. The colorimetric readings were taken after the addition of resazurin dye [12].

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

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

blue exclusion, and the macrophages were seeded in 24-well plates (Nunc, Roskilde, Denmark) with complete RPMI at a density of approximately 5×10^5 cells/well and incubated overnight. The THP-1 cells were differentiated by 50nM phorbol 12-myristate 13-acetate (PMA) induction to achieve macrophage phenotypes and were incubated at 37°C/48-72h/5% CO_2. After 48 h of activation, the THP-1 macrophages were infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 1:10 [12] and were incubated for 2 h at 37°C / 5% CO_2. The medium containing the mycobacteria was discarded, macrophage monolayers were washed twice with 3 mL of PBS (+Ca^{2+} + Mg^{2+}) to remove the free bacteria and replenished with fresh complete RPMI. Sets of triplicate wells were lysed (0.05% SDS) at specific time-points and enumerated to estimate the numbers of intracellular Mtb 2hr post-infection [13,14,15]. The phosphate-buffered saline washed monolayers were lysed by adding 1 mL of water plus 0.05% sodium dodecyl sulfate (SDS) for 5 min. The lysate was serially diluted and plated onto Middlebrook 7H11 agar plates (Difco Labs) for cfu enumeration (read after 3 to 4 weeks).

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

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

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

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

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

To generate spontaneous mutants resistant to FNDR-20081, 10^8 cfu of log phase cells (OD_{600} of 0.8-1.0) were plated out onto 7H11 agar containing 5×, 10× and 20× MIC of the compound. To confirm resistance, putative mutants were grown in liquid 7H9 media, in the absence of the compound, to log phase. The cells were spotted as 10 µL of 10^4, 10^3, 10^2 and 10^1 cfu on to 7H11 agar containing 5× MIC of FNDR-20081. Resistant mutants that grew on this plate were subjected to whole-genome sequencing (WGS) to determine mutations conferring resistance. Cells were grown to log phase in 50 mL liquid media, containing 3× MIC of FNDR-20081, and the genome purified. WGS and the alignment to the reference genome of *M. bovis* BCG Pasteur 1173P2 (accession number: NC_008768.1) was completed by MicrobesNG.

Target Gene Over-Expression: Target genes were cloned into the plasmid pMV261, under the control of a constitutive promoter, in the presence of KAN (50 µg/mL) as selection marker. Compounds were serially diluted 2-fold, to obtain a range covering the MIC at 50× the desired concentration, and 2 µl was transferred across to 96-well Greiner black bottomed plates, leaving a single row moisture barrier around the outside of the plate. BCG or Mtb strains, containing the pMV261 plasmids, were grown to mid-log and diluted to 1.5×10^6 colony forming units (CFU)/mL. 98 µl of cells per well, was added to the assay plates, which were sealed and incubated at 37˚C in a CO_2 incubator. After 7 days, 30 µl of a 0.02% (w/v) solution of resazurin and 12.5 µl of 20% tween-80 was added and the plates were incubated overnight before measuring fluorescence (excitation at 530 nm and emission at 590 nm) using a POLARstar Omega plate reader (BMG Labtech.). MICs of target gene over-expressing strains were compared with a strain carrying a pMV261 empty vector. This method was also used to compare the liquid MICs of the spontaneous mutants generated to those of the wild type (WT) strain of BCG.

Drug Metabolism and Pharmacokinetics of FNDR-20081:

Microsomal stability: Microsomal stability was performed using human and mouse liver microsomes. The final composition of the assay included 1 µM of FNDR-20081and the final concentration of DMSO 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 mM $MgCl_2$, 1.0 mM $NADP^+$). The compound was incubated with human and mouse liver microsomes with and without cofactors. The reaction mixture was removed at specified time points (0, 15, 30, 60 and 120 min) and the reaction was stopped by addition of ice-cold acetonitrile. The samples were extracted in presence of internal standard (Haloperidol) and were analyzed using LC-MS/MS. After the specified incubation period, percent of the remaining test/control compound was calculated with respect to the peak area ratio at time 0 min [7].

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

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

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

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

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

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

Dose response in chronic infection model. BALB/c mice were infected via aerosol inhalation in a Madison chamber calibrated to deliver 100 CFU/mouse lung [13]. Infected mice were housed in isolators (Allentown technologies, USA) during the entire period of experimentation. Treatment began 4 weeks post infection. FNDR-20081 was administered once daily, by oral gavage in a vehicle (5% (v/v) DMA + 5% (v/v) Tween 80 + 5% (v/v) propylene glycol + 85% (v/v) sterile water). Three doses, 10, 30 and 100mg/kg body weight were given 7 days a week, for a period of 4 weeks. RIF was used as a positive control. On completion of dosing, animals were sacrificed 48 hours later by CO_2 narcosis, lungs were removed, homogenized, and plated for enumeration of CFU/lung on Middlebrook 7H11 media plates supplemented with OADC and PANTA (BD-245114). The plates were incubated at 37°C with 5% CO_2 for 3 weeks prior to reading bacterial CFU counts.

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

Intracellular efficacy of FNDR-20081: The intracellular mycobacterial killing rates of FNDR-20081 and the control drug rifampicin were determined [12] by nonlinear regression analysis (95% confidence limits). Inhibitory curves were generated by plotting the $\log_{10}$ cfu/mL against the Day-0, 3, 5 and 7. The Emax observed was 1.5 $\log_{10}$cfu/mL (Figure 4)

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

Target identification of FNDR-20081:

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

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

FNDR-20081 Spontaneous Resistance Mutants in *M. bovis* BCG: A systematic approach to target identification was adopted by the generation of spontaneous mutants. The MIC of FNDR-20081, for *M. bovis* BCG grown on solid media, was 6.25 µg/mL. Spontaneous resistant mutants grew at 5×, 10× and 20× MIC of FNDR with frequencies of resistance (FoR) of 7×10^{-8}, 12×10^{-8} and 19×10^{-8}, respectively. The genomes of four resistant mutants (one from 10× and three from 5× MIC) were sequenced and aligned to the genome of the parental strain to determine the mutations that could give rise to resistance. All four mutants carried mutations in the *marR* regulator (BCG_0727, corresponding to Rv0678 in Mtb): a mutation in three of the mutants introduced a stop codon thereby interrupting gene expression; the other mutant had an insertion, resulting in a frame shift.

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

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

Drug Metabolism and Pharmacokinetics:

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

In addition, the CYP3A4 inhibition assay with FNDR-20081 revealed no CYP3A4 liability and the IC_{50} was >25 µM. Other compounds in the series demonstrated CYP3A4 inhibition at <2µM.

Oral pharmacokinetics of FNDR-20081: Mean plasma concentrations of FNDR-20081 at 30 mg/kg and 300 mg/kg doses are shown in Figure 7. Orally bioavailable, a saturable absorption was observed for the compound (Cmax) of 7.4 µg/mL for the 30 mg/kg dose and 13.8 µg/mL for the 300 mg/kg dose group. There was a dose proportional increase in plasma exposures. AUC_{inf} for the 30 mg/kg dose was 11.9 h*µg/mL and for the 300 mg/kg bw was 103.775 h*µg/mL.

Pharmacokinetics in infected animals:

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

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

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

Discussion

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

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

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

against FNDR-20081 do demonstrate cross-resistance to CLT. But interestingly, in the other three subsequent spontaneous mutants to FNDR-20081, that had no mutations in *marR* and were not cross-resistant to CLT, mapped to BCG_3742, corresponding to Rv3683 in Mtb. Rv3683 is a metallophosphoesterase, while non-essential by transposon site hybridization (TraSH) [43], the location of Rv3683 to a penicillin binding protein (PBP) on the genome could form part of a more significant interaction with this protein and peptidoglycan biosynthesis. Additional studies will be required to further validate Rv3683 also as a target of FNDR-20081.

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

Another emerging concept that could impact treatment of MDR-TB is multitarget therapy [4]. "Multi-targeting" therapy, with a single drug is epitomized to be a preferred approach over conventional mono-targeting [20,44-48]. Many of the successful anti-TB regimen drugs (e.g., isoniazid, ethambutol, pyrazinamide and Pretomanid) [48] as well as another drug in the pipeline, ethylene diamine drug, SQ109 (an uncoupler inhibiting two distinct proteins involved in cell wall and menaquinone biosynthesis (Mmpl3, MenA and MenG, and ATP biosynthesis proteins) exhibit multi-targeting phenomenon [48-50]. Among the existing drugs, coumarins (e.g., Novobiocin), inhibit DNA topoisomerases along with Fad24 and FtsZ [49]. Such multi-targeting drugs are powerful tools to tackle multi-drug-resistance. FNDR-20081 also probably hits multiple targets within the cell wall such as regulator of *mmpS5-mmpL5* and a metallo-phosphoesterases. However, more intricate studies are required to confirm the targets with identification of the precise ligand binding site. It may provide an opportunity to develop new combinations against drug resistant Mtb.

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

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

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

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

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

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

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

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

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Declaration of competing interest

The authors declare no competing interests.

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Tables.

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
Invitro combination studies with First line drugs				
1	FNDR-20081	Isoniazid	1.29	Additive
2	FNDR-20081	Rifampicin	1.94	Indifference
3	FNDR-20081	Ethambutol	0.54	Synergy
Invitro combination studies with Second line drugs				
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
In-vitro combination studies with New TB drugs				
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

TABLE 2: Serum/ Plasma protein binding.

MIC Mtb (µg/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

TABLE 3: FNDR-20081 activity: **a.** under Low pH (1.2 $\log_{10}$ cfu/mL), Nutrient starvation (0.7 $\log_{10}$ cfu/mL), stationary phase (0.2 $\log_{10}$ cfu/mL), **b**. Kill against replicating *vs.* non-replicating Mtb

a.

Conc. µg/ml	**Emax** (Log_{10} 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

b.

Condition	**Emax** (Log_{10} 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

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

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

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

Figure Legends

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

Figure 2. MIC of FNDR-20081 against 61 Mtb strains of different resistance patterns [Blue bar=WT (1) Mtb strain, red bars=XDR(5), yellow bars=MDR(33), purple bars=SDR (15), and green bars=drug sensitive (8) Mtb strains] grouped under two categories: low MIC range (≤4µg/mL) and high MIC range (up to 16µg/mL). Total ≥92% strains were sensitive to FNDR-20081. n= number of isolates under respective low MIC or high MIC categories.

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

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

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

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

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

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

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

Figures:

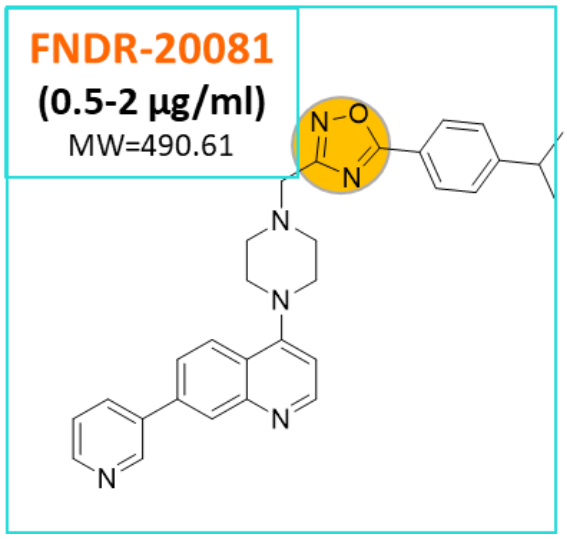


FIG 1*: FNDR-20081.Oxadiazole--piperazine-quinoline in tandem (4-{4-[5-(4-Isopropyl-phenyl)-[1,2,4] oxadiazol-3-ylmethyl]-piperazin-1-yl}-7-pyridin-3-yl-quinoline). Mtb MIC 0.5µg/ml (1.02µM).*

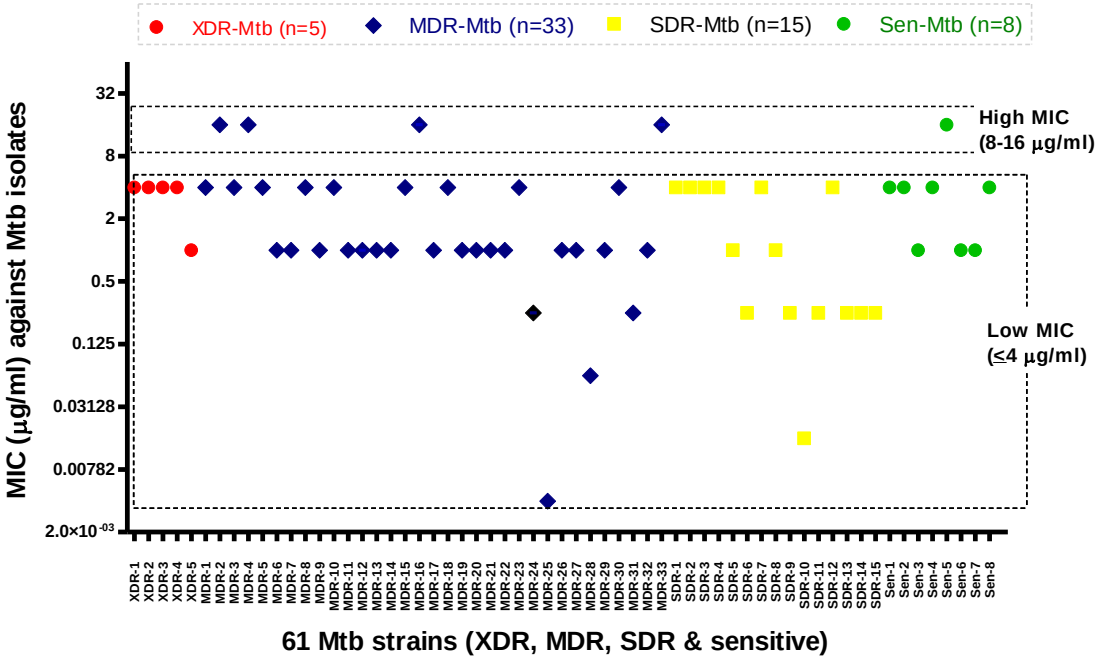
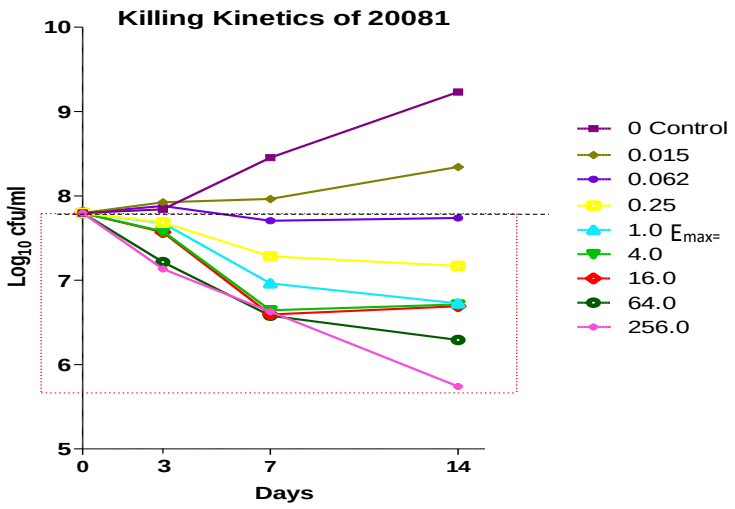


FIG 2. *Total 61 Mtb strains from different resistance patterns [XDR (5), MDR (33), SDR (15), drug sensitive (8)] grouped under into two categories: Low MIC range (≤4µg/mL) and High MIC range (up to 16µg/mL). Total ≥92% strains were sensitive to FNDR-20081.*



KK Emax	D0	D3	D7	D14
20081	0.0	0.7	1.2	2.1

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

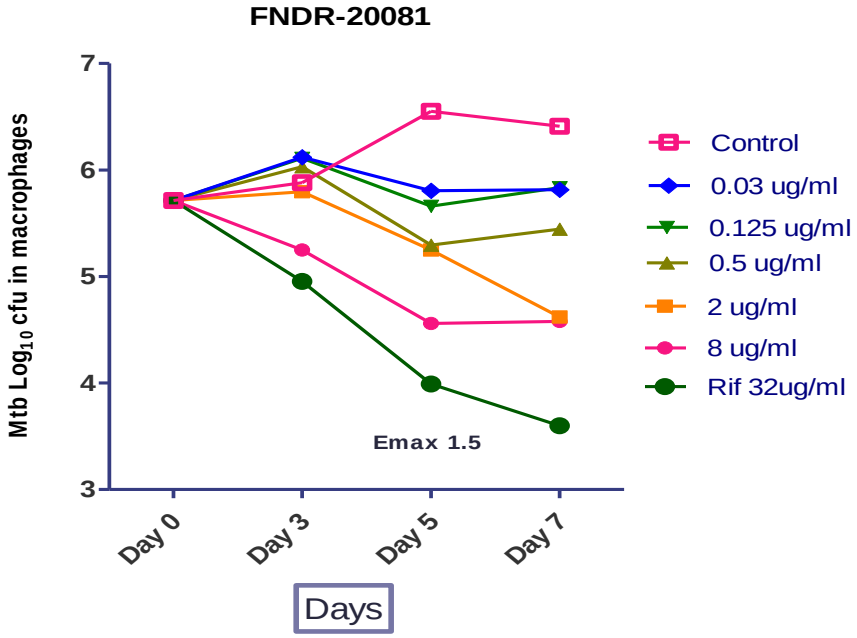


FIG 4. *Intracellular efficacy Emax=1.5 $\log_{10}$ cfu/mL*

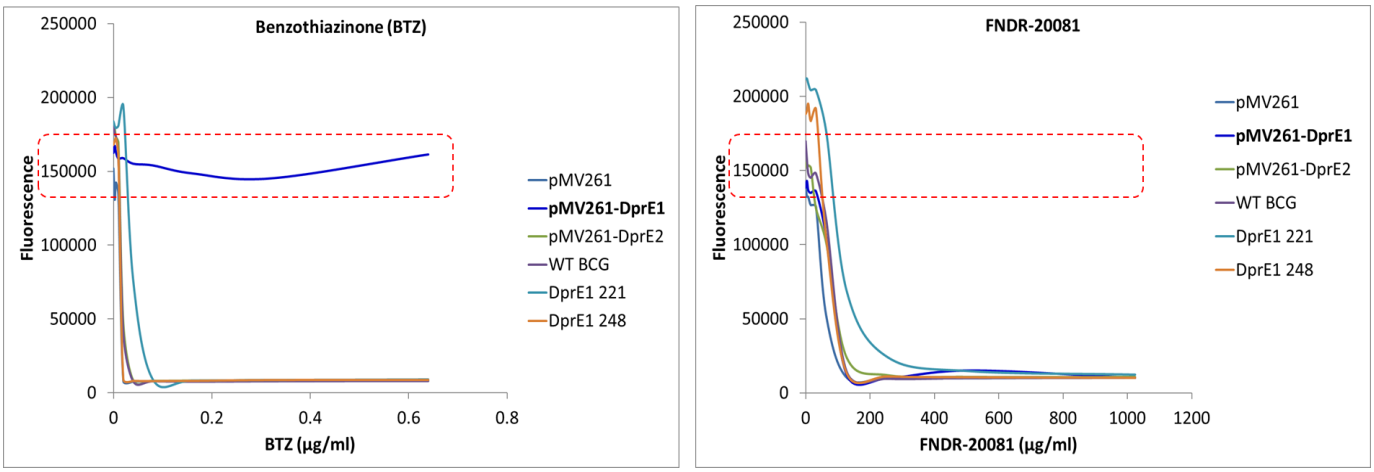


FIG 5. *Over-expression studies of DprE1 in M. bovis BCG. Over-expression of DprE1 confers a greater than 16-fold increase in resistance to BTZ043, but no resistance to FNDR-20081.*

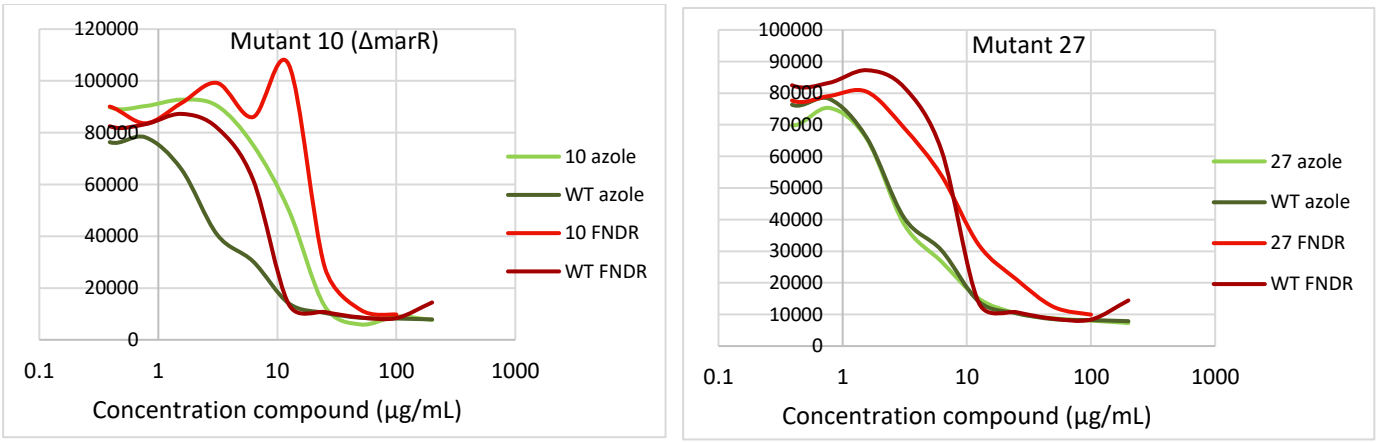
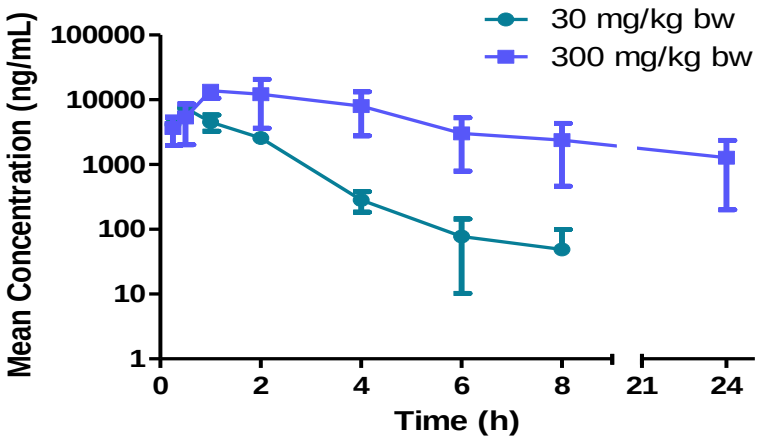


FIG 6. *Cross-resistance of FNDR-20081 spontaneous mutants to clotrimazole. The MIC of the mutants generated to FNDR-20081 was examined in liquid culture, for clotrimazole and FNDR-20081, using a 2-fold serially diluted range of drugs in a 96 well plate. Live bacteria were identified by measuring fluorescence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

a.

Parameter		FNDR-20081
Liver microsomal stability (%)	MLM	**1.22**
	HLM	**40.1**
CYP 3A4 inhibition	IC_{50} (µM)	**>25**
HepG2 cell toxicity (%)	100 µM	**38.7**



b.

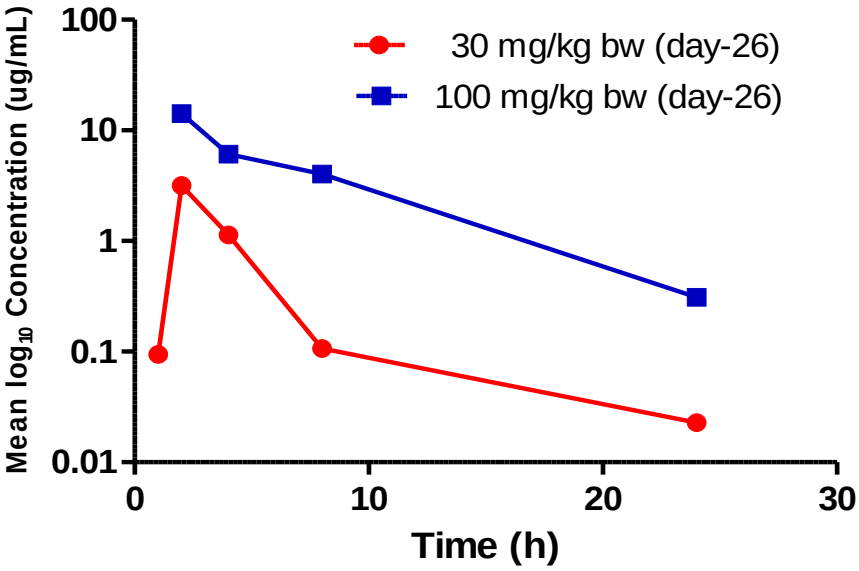
Parameters	30 mg/kg, po	300 mg/kg po
C_{max} (µg/mL)	7.4	13.8
T_{max} (h)	0.5	1
AUC_{last} (h*µg/mL)	11.9	85.3
AUC_{inf} (h*µg/mL)	11.9	103.8
AUC_{extrap}(%)	0.59	17.78
MRT_{last} (h)	1.38	6.64

c.

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

a.

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



b.

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

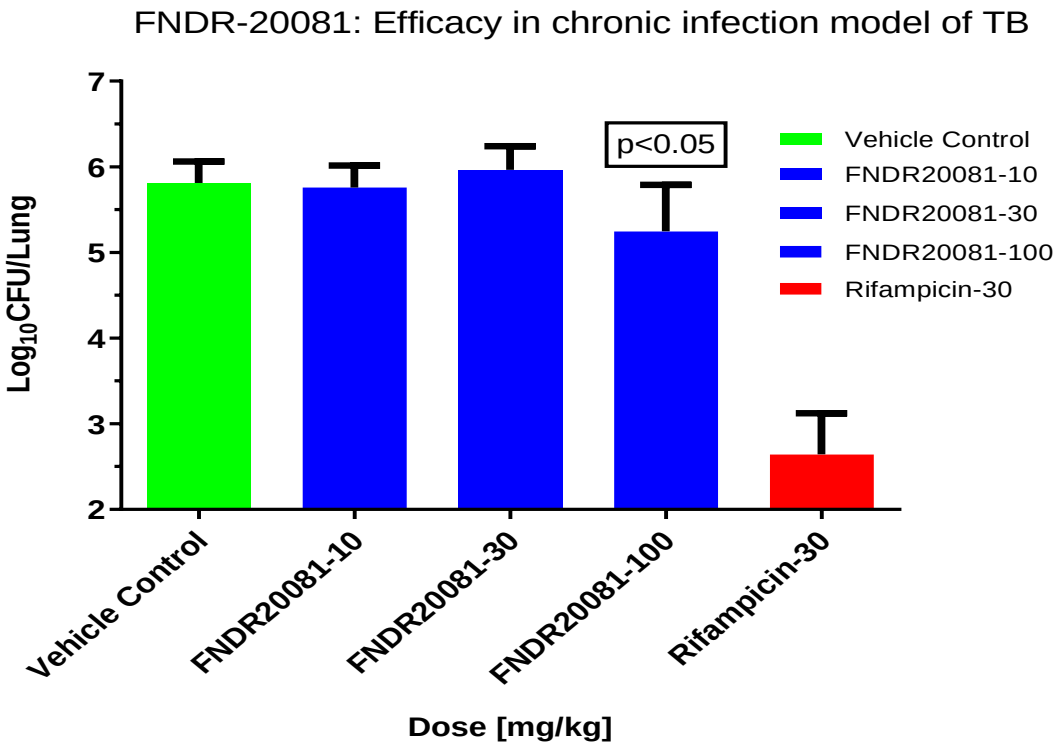


FIG 9. *In-vivo efficacy of FNDR-20081 in BALB/c mice demonstrated significant (P<0.05) 0.56 log_{10} CFU/lung reduction.*

954 Supplementary Table S1:

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

CHROM	POS	TYPE	REF	ALT	EVIDENCE	FTY	STRA	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE	PRODUCT	FNDR22	FNDR23	FNDR27	number_sa	ples_with_variant
NC_008769	1E+06	complex	CG	GC	GC:8 CG:0										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		PE family protein	C:5 G:0			1	
NC_008769	2E+06	complex	CG	CCGGC	CCGGC:7 CG:	CDS	-	666/2690	222/895	disruptive_inframe_insertion & synonymous_variant c.666delCinsGCCG p.Thr222_Val223insPro	BCG_1799c	Rv1759c	hypothetical protein	CCGGC:7 CG:	CCGGC:7 CG:	CCGGC:6 CG:	3	non essential gene by Himar1-based transposon mutagenesis in H37Rv strain (see Sassetti 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.Thr177Thr	BCG_2507c		LuxR family transcriptional regulator	A:36 T:0	A:42 T:0	A:33 T:0	3	
NC_008769	3E+06	ins	TTA	TATA	TATA:56 TTA	CDS	+	75/1743	25/580	frameshift_variant c.74_75insA p.Phe25fs	BCG_2963		long-chain-fatty-acid--AMP ligase FadD28		TATA:56 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		cation:proton antiporter	T:26 C:0	T:46 C:0	T:31 C:0	3	
NC_008769	4E+06	ins	GGC	GGCCGC	GGCCGC:17 (	CDS	-	1296/1419	432/472	conservative_inframe_insertion c.1296_1297insGCG p.Ala432dup	BCG_3499c	Rv3433c	bifunctional ADP-dependent NAD(P)H-hydrate dehydratase/NAD(P)H-hydrate epimerase	GGCCGC:17 (	GGCCGC:13 GGC:0		2	non essential gene by Himar1-based transposon mutagenesis in H37Rv strain (see Sassetti et al., 2003) disruption causes growth advantage in vitro
NC_008769	4E+06	ins	GGC	GGTCGC	GGTCGC:18 (	CDS	+	783/786	261/261	disruptive_inframe_insertion c.782_783insTCG p.Gly261_Ter262insArg	BCG_3517	Rv3451	cutinase family protein	GGTCGC:18 (	GGTCGC:21 (	GGTCGC:9 G(	3	KO is more resiliant 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.Thr598Ala	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	3	non essential gene by Himar1-based transposon mutagenesis in H37Rv strain (see Sassetti 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	glpK	glycerol kinase GlpK	G:34 C:3	G:35 C:0	G:23 C:0	3	
NC_008769	705623	complex	GTGG	ATGC	ATGC:7 GTG(	CDS	-	1176/3912	391/1303	synonymous_variant c.1173_1176delCCACinsGCAT p.393	BCG_0623c		PE family protein	ATGC:7 GTGG:0			1	