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Abstract

Despite being one of the first antitubercular agents identified, isoniazid (INH) is still the most prescribed drug for prophylaxis and Tuberculosis (TB) treatment and together with rifampicin the pillars of current chemotherapy. A high percentage of isoniazid resistance is linked to mutations in the pro-drug activating enzyme KatG, so the discovery of direct inhibitors (DI) of the enoyl-ACP reductase (InhA) has been pursued by many groups leading to the identification of different enzyme inhibitors, active against Mycobacterium tuberculosis (MtB), but with poor physicochemical properties to be considered as preclinical candidates. Here, we present a series of InhA DI active against multidrug (MDR) and extensively (XDR) drug resistant clinical isolates as well as in TB murine models when orally dosed that can be a promising foundation for a future treatment.

1. Introduction

Although most of the drugs for Tuberculosis were discovered more than 50 years ago, TB accounts for about 1.4 million deaths every year (Zumla et al., 2013). The majority of the TB cases are treatable with the current long and complex regimen of drugs, although lack of adherence due to adverse effect is not unusual, leading to suboptimal responses and rising incidences of M(X)DR cases worldwide. In the last forty years only two new drugs have been approved for the treatment of MDR-TB under specific conditions: bedaquiline and delamanid (Andries et al., 2005; Thakare et al., 2015). Thus, there is a critical need for the development of drugs with shorter, simpler regimens as well as novel mechanisms of action that can be used for treatment of drug-resistant forms of the disease.
Both target-based (Makarov et al., 2009) and phenotypic screening (Ballell et al., 2013; Lechartier et al., 2014; Mak et al., 2012; Pethe et al., 2010) approaches have been employed for the identification of anti-tubercular drug leads. While a limited but significant number of examples exist for the latter, (Abrahams et al., 2012; Makarov et al., 2009; Remuinan et al., 2013) target-based approaches have encountered very limited success as previously demonstrated in the antibacterial field (Abrahams et al., 2012; Payne et al., 2007). Rather than invalidating the approach per se, this situation highlights the disconnection between concepts like genetic validation of target essentiality and the amenability of that target for drug discovery. A deeper understanding of system biology and the mechanisms underlying antibiotic killing are important for the discovery of new antimicrobial therapies through target-based approaches. Additionally, for reasons that are not always obvious, some targets are clearly more chemically tractable than others. For example, protein and cell wall biosynthesis and DNA gyrase have delivered multiple classes of published leads and marketed drugs, whereas there are no known inhibitors for many other essential gene products, despite a long history of antibacterial research (Kohanski et al., 2010). In the antitubercular field, only a very limited number of targets such as InhA, RpoB, DNA Gyrase, ATP synthase and DprE1 have been shown to be behind the action of potent bactericidal drugs or promising leads.

Isoniazid is a frontline anti-TB drug targeting InhA and is an essential component of TB treatment regimen. Despite the seemingly simple structure of INH, its mode of action has remained elusive for many years (Vilcheze and Jacobs, Jr., 2007). INH penetrates the tubercle bacilli by passive diffusion and is activated by the bacterial anti-oxidant enzyme KatG to a range of reactive species and isonicotinic acid. Relevant reactive species form adducts with nicotinamide adenine dinucleotide (NAD), which are able to
interfere with NAD-utilising enzymes, primarily the enoyl-ACP reductase encoded by the \textit{inhA} gene, leading to the blockage of mycolic acid synthesis and delivering the lethal blow to \textit{Mycobacterium tuberculosis}. The dependency on KatG activation for INH-mediated killing is also the source of the main clinical weakness associated with the use of INH, as between 40-95\% of INH-resistant MTB clinical isolates have mutations in \textit{katG}, leading to decreased activation of INH to its active form (Hazbon et al., 2006; Seifert et al., 2015). While mutations are also detected in clinical isolates in the \textit{inhA} promoter region, these can be successfully treated in most instances by increasing the dose of isoniazid.

Different classes of direct InhA inhibitors have been identified previously using high-throughput screening, Encoded Library Technology and \textit{in silico} design strategies (Lu et al., 2010; Manjunatha et al., 2015; Pan and Tonge, 2012; Shirude et al., 2013; Sink et al., 2015; Vilcheze et al., 2011; Encinas et al., 2014). Additionally, natural product pyridomycin has been found to operate via InhA inhibition (Hartkoorn et al., 2012; Lu et al., 2010). Most of these tended to show a lack of correlation between enzymatic inhibition and whole cell activity, have moderate potencies, narrow selectivity windows or poor absorption, distribution, metabolism, and excretion (ADME) properties, making them unsuitable for further progression as drug leads.

GlaxoSmithKline (GSK), under the sponsorship of the TB Alliance has carried out a screen against InhA using the GSK compound collection and has identified the thiaiazole series as the most promising anti-tubercular family. In this study we present the novel and selective lead compound and its attractive anti-tubercular properties.
2. Materials and Methods

The human biological samples were sourced ethically and their research use was according to the terms of the informed consent.

All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals.

2.1. Compound Synthesis

GSK613 and GSK625 were obtained from commercial sources. GSK693 was synthesized as described in the patent (Castro-Pichel et al., 2012). Optical rotations were measured on a Rudolph AUTOPOL V polarimeter at room temperature using a cell of 0.5 dm. $^1$H NMR spectra were recorded on a Bruker DPX 400MHz NMR spectrometer. Measurements were made at a temperature of 295 K, and are reported in ppm using tetramethylsilane or solvent as an internal standard (DMSO-d$_6$ at 2.50 ppm). The coupling constants ($J$) are given in Hz, and the splitting patterns are designated as: s, singlet; bs, broad singlet; d, doublet and m, multiplet. $^{13}$C NMR spectra were recorded on a Bruker DPX 400 spectrometer at 295 K, and are reported in ppm using solvent as an internal standard (DMSO-d$_6$ at 39.5 ppm). Analytical purity was $\geq$95%, as determined by $^1$H-NMR and HPLC analysis. Positive ion mass spectra were acquired using a QSTAR Elite (AB Sciex Instruments) mass spectrometer, equipped with a turbospray source, over a mass range of 250 – 700, with a scan time of 1 sec. The elemental composition was calculated using Analyst QS 2.0 software.

$^{(S)}$-1-(4-methylthiazol-2-yl)-1-(5-((1-((2-methylthiazol-4-yl)methyl)-1H-pyrazol-3-yl)amino)-1,3,4-thiadiazol-2-yl)ethanol (GSK693): $[\alpha]_{D}^{20} = +119$ (c = 1, MeOH). $^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ ppm 10.93 (bs, 1H, -NH), 7.75 (d, $J = 2.20$ Hz, 1H), 7.38 (bs, 1H), 7.33 (s, 1H, -OH), 7.28-7.29 (m, 1H), 6.03 (d, $J = 2.27$ Hz, 1H), 5.32 (s,
2H), 2.68 (s, 3H), 2.37 (s, 3H), 2.02 (d, J = 7.03 Hz, 3H). $^{13}$C-NMR (100 MHz, DMSO-$d_6$): δ ppm: 17.38, 19.18, 28.77, 51.32, 74.67, 94.29, 115.29, 117.15, 132.43, 148.65, 151.73, 152.42, 156.50, 164.66, 166.28, 175.79. HRMS (m/z): [M+H]$^+$ calcd. for C16H17N7OS3, 420.0730; found, 420.0717.

2.2. Bacterial strains and culture

*Mycobacterium tuberculosis* H37Rv, *Mycobacterium smegmatis* mc²155 (Snapper et al., 1990a) and *Mycobacterium bovis* BCG Pasteur (Institut Pasteur) were grown at 37ºC in Middlebrook 7H9 broth (Difco) supplemented with 0.025% Tween 80 and 10% albumin-dextrose-catalase (ADC) or on Middlebrook 7H10 plates supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). Cell-free extracts were done in 7H9 (Difco) supplemented with 100 ml of 10X AS solution (5% albumin solution in salt: 10 mg albumin, 1.7 mg NaCl in 200 ml water), 2.5 ml of 10% Tween 80 solution and 0.1% carbon substrate (acetamide, succinate, or glucose). *Escherichia coli* DH5α was grown in LB broth (LB).

2.3. DNA manipulation, plasmids and transformation

General molecular biology procedures were used as described previously (Green and Sambrook, 2012) or following the manufacturer instructions. *Escherichia coli* DH5α, *M. smegmatis* mc²155 and *M. bovis* BCG competent cells were prepared for electroporation as described previously (Goude et al., 2015; Green and Sambrook, 2012).

2.4. Enzymatic purification of InhA

The plasmids were transformed into BL21(DE3) *E.coli* cells for protein overexpression. Cells carrying InhA over-expression plasmid were cultured overnight in LB broth media together with 100 μg/ml ampicillin at 37ºC with continuous shaking at 220 rpm. Then, a 1 % dilution of the inoculum was made (10 ml of the starter culture into 4 x 1 liter) in
LB broth media with 100 μg/ml ampicillin and flasks were incubated till the OD$_{600}$ reached 0.7. Cells were induced with 0.5 mM IPTG at 30-32°C for 3 hours, harvested and resuspended for lysis in 100 ml total volume of 10 % glycerol, 25 mM Tris pH 8.0 and 2 mM DTT (freshly made) at 4°C. Cells were then sonicated 4 x 15 seconds at maximum amplitude with 45 seconds incubation on ice between pulses, and finally centrifuged at 30,000 g at 4°C for 1 hour.

The supernatants were loaded on a 6 ml Resource Q columns which were pre-equilibrated with 25 mM Tris HCl pH 8.5, 2 mM DTT. 2.5 ml fractions were collected over 20 column volumes (gradient of 0-200 mM NaCl, 25 mM Tris HCl pH 8.2, 2 mM DTT). The fractions were run out on a SDS-PAGE gel and stained with Coomasie. The most concentrated ones were selected and pooled to run on a Superdex 16/60 SEC to help decontaminate. The column was equilibrated with 25 mM Tris HCl pH 8.0, 2 mM DTT and the pooled fractions were applied to the column at 0.12 ml/min. 1 ml fractions were collected in the following buffer: 20 mM Tris HCl pH 8.0, 2 mM DTT, 0.15 M NaCl. The column was run overnight, the fractions were checked by SDS-PAGE and activity was verified by enzymatic assay.

2.5. InhA biochemical assays

The trans-2-enoyl-ACP reductase enzyme of *M.tuberculosis* catalyzes the last step in the elongation cycle of the FAS-II pathway and reduces the 2,3 double bond of trans-2-enoyl-ACP in a NADH dependent manner. High Throughput Screening (HTS) and Led Optimization (LO) biochemical assays are based in the oxidation of the cofactor in the presence of dodecenoyl-CoA.

InhA inhibition by file compounds in a high throughput format was assessed using a substrate-induced quenching (SIQ) assay as described previously (Vazquez et al., 2006). Reaction mixtures (3μl) containing 100 μM test compound, 150 μM dodecenoyl-CoA,
100 µM NAD+, 30 µM NADH, 10 nM resorufin, 0.2% w/v pluronic acid F-127, 10 nM resorufin, 0.01% BSA, 1% DMSO and 1.25 nM InhA in 30 mM PIPES buffer at pH 6.8 were incubated in glass-bottom 1536-well plates for 1 hour at 20 ºC and 95% relative humidity. Plates were then read in a confocal microscope (4-Channel Reader, Evotec Technologies) using 1D-FIDA settings with beamscanner, Nd:Yag 532 nm excitation with a laser power of 150µW for 200 ms/well. Confocal FLINT data from each screening plate were normalized against control wells containing reaction mixtures in the absence of test compounds (high controls) and in the absence of enzyme (low controls). Assay quality was monitored for each plate using the Z’ factor (Zhang et al., 1999) and the inhibition observed in additional control wells containing a known inhibitor at its IC50. In order to identify and discard signal quenchers each plate was pre-read before addition of enzyme.

Enzymatic activity to support LO phase was measured fluorimetrically by following NADH oxidation at λexc=340 nm and λem= 480 nm, using 50 µM NADH and 50 µM 2-trans-dodecenoyl-CoA (DDCoA) as substrates. Dose-response experiments to determine IC50 were performed using 5 nM InhA, percentage of remaining enzymatic activity (%AR) at different compound concentrations were calculated with the next formula [%AR = 100*((sample – control 2) / (control 1- control 2))] where sample is the enzymatic activity for each compound concentration, control 1 is enzyme activity in absence of any compound and control 2 is NADH oxidation in absence of enzyme; IC50 were calculated fitting %AR to a 2 parameter equation [%AR = 100% / (1 + (compound conc / IC50)^s)] where s is a slope factor, IC50 were calculated using GraFit 5.0.12 software (Eritacus Software Ltd). All reactions were run in 30 mM PIPES buffer, pH 6.8, at 25 ºC.
2.6. Crystallization, data collection and processing

The InhA:GSK625 complex was crystallized using the hanging drop method. 0.2 mM InhA, 2 mM NAD$^+$, and 2 mM GSK625 that was dissolved in 100% DMSO were incubated at room temperature for ~20 min and screened against sparse-matrix crystallization conditions. The crystals were obtained in 1.1 M sodium malonate, 0.1 M HEPES pH 7.0, 0.5% (v/v) Jeffamine ED-2001 pH 7.0.

The diffraction data were collected at home source, at 120 K under cryoprotection by a Rigaku Raxis detector coupled to an X-ray generator with a rotating copper anode ($\lambda = 1.541$ Å). Data was processed by Denzo and Scalepack software packages (Otwinowski and Minor, 1997). Crystals of the InhA:NAD$^+$:GSK625 complex belonged to the $P2_12_12_1$ space group with four molecules in the ASU. InhA:GSK625 structure was solved by molecular replacement using the PDB entry 1ENY as a search model. The structure was built by Coot and further refined by Phenix (Adams et al., 2010) yielding a final model with $R_{\text{work}}=20\%$, and $R_{\text{free}}=23\%$. Data processing statistics are given in Supplementary Table 1, PDB ID 1BVR.

2.7. Cloning and overexpression of InhA

Double digestion with BglII and BamHI liberates M. smegmatis and M. tuberculosis inhA genes from plasmids pATB15 and pATB14 (Parish et al., 1997) respectively. The 918 and 917 bp fragments were cloned into pATB45 previously linearized by BamHI digestion. Ligations were performed into E. coli DH5α. The final plasmids were verified by enzymatic fragments analysis and both inhA genes were sequenced. Sequencing was carried out with using the dideoxy chain-termination procedure and reagents from Applied Biosystems (dRhodamine Terminator Cycle Sequencing Ready Reaction kit) in an ABI Prism 310 automated DNA sequencer (Applied Biosystems). Electroporation of M. smegmatis mc²155 and M. bovis BCG were carried out according to the method of
(Snapper et al., 1990b) and transformants were selected on Middlebrook 7H10 plates supplemented with 10% OADC and hygromycin 50 µg/ml. Transformants verifications were made by colony PCR by using the PuRe Taq Ready-To-Go PCR Beads kit (GE Healthcare Life Sciences) with the primers colony PCR 1 and 2. The sequences of the primers were as follows: Primer colony 1, 5'-AATCCAAAGTCCAAAAAGGGG-3'; Primer colony 2, 5'-CCACCACCCGATAAGAAGGGG-3'. The following thermocycler parameters were used: an initial denaturation step of 5 min at 94ºC, followed by 35 cycles of 30 s at 94ºC, 30 s at 62ºC and 3 min at 72ºC and ending with a final elongation step of 10 min at 72ºC.

Protein expression levels were followed by SDS-PAGE. Cell free extract were obtained from M. smegmatis mc²155 and M. bovis BCG by growing them in 500 ml Middlebrook 7H9 broth with 0.025% Tween 80 and 10% ADC (11 conical flask) to log phase (OD 600 nm ≈0.6 ) harvested by centrifugation (5000 rpm for 5 min) and resuspended in 5 ml sterile distilled water. 0.5 ml of M. smegmatis mc²155 cells was used to inoculate 250 conical flasks containing 100 ml minimal medium with either acetamide 1%, succinate 0.1%, or succinate 0.1% plus acetamide 1%. 0.5 ml of M. bovis BCG cells was used to inoculate 250 ml conical flasks containing 100 ml minimal medium with either acetate 0.1%, acetate 0.1% plus acetamide 1%, ADC 10% or ADC 10% plus acetamide 1%. Cultures were incubated at 37ºC and harvested after 24 hours (M. smegmatis mc²155) or 7 days (M. bovis BCG).

Cell-free extracts were prepared from both cultures using the Mini-BeadBeater (Biospec Products): 50 ml bacterial cells, resuspended in 1 ml 50 mM HEPES/KOH pH 7.5, 10 mM MgCl₂, 60 mM NH₄Cl, 10% (v/v) glycerol, 5 mM 2-mercaptoethanol, were added to 0.5 ml of 0.1mm sterile glass beads and shaken for 1 min three times, incubating the samples on ice 1 min between pulses. The supernatant was recovered after
centrifugation. Cell-free extracts were analysed by SDS-PAGE (Laemmli, 1970); protein concentration was determined using the BCA protein assay (Pierce).

2.8. Isolation and characterization of *M. tuberculosis* H37Rv GSK625 resistant mutants

GSK625-resistant *M. tuberculosis* H37Rv mutants were isolated by plating 10^8 CFU on Middlebrook 7H10 plates supplemented with 10% OADC containing the compound at 20 µM (20xMIC). After 4 weeks of incubation at 37ºC, colonies were identified and purified through several passages in inhibitor-containing plates. Single colonies were used to amplify and sequence *inhA* alleles. DNAs of the 17 resistant mutants were extracted from liquid cultures grown in Middlebrook 7H9 broth supplemented with 0.025% Tween 80 and 10% ADC. 1 ml of culture was centrifuged, the pellet resuspended in 500 µl of distilled water and incubated at 90ºC for 1 h, and then filtrated by using 0.22 µm filters. 5 µl of the supernatant was used as a source of genomic DNA for amplification of the *inhA* gene. PCRs of the entire gene were performed for each mutant by using the PuRe Taq Ready-To-Go PCR Beads kit (GE Healthcare Life Sciences). A 988 bp region was amplified with the primers Forward (5'-CAGCTTCCTGGCTTCCGAG-3') and Reverse (5'-TAACGTTCCTCCAGGAACGG-3'), and PCR conditions were: an initial denaturation step of 5min at 94ºC, followed by 40 cycles of 30s at 94ºC, 30s at 62ºC and 3min at 72ºC and ending with a final elongation step of 10 min 72ºC). PCR products were purified and sequenced using the dhdamidine Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in an ABI Prism 310 automated DNA sequencer (Applied Biosystems).

2.9. Complementation experiments

The whole *fabG1-inhA-hemH* operon from wild type H37Rv and mutants carrying the four mutant alleles (M103V, G96V, M103I, M103T) were cloned in the shuttle vector
pSUM36 (Ainsa et al., 1996). *M. tuberculosis* H37Rv was transformed with the five plasmids and transformants were selected using kanamycin and verified by colony PCR using the PuRe Taq Ready-To-Go PCR Beads kit (GE Healthcare Life Sciences). The sequences of the primers were as follows: pSUM36_2, 5'-GTTGTGTGGAAATTGTGAGCG-3'; InhArevi, 5'-GAAACGCGATCGACGAGTCGG-3'. The following thermocycler parameters were used: an initial denaturation step of 9 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 64°C and 3 min at 72°C and ending with a final elongation step of 10 min at 72°C. Positive transformants were used MIC determination.

2.10. MIC determination against mycobacteria

The antitubercular activity against extracellular of intracellular *Mycobacterium* strains was performed as previously described (Blanco-Ruano et al., 2015).

2.11. MIC against clinical strains

The BACTEC MGIT 960 System (Becton Dickinson) was used to MIC determination in clinical isolates (Institute Carlos III and Hospital Val d’Hebron) following the manufacturer instructions.

2.12. General antimicrobial activity assay

Whole cell antimicrobial activity was determined by broth microdilution using the Clinical and Laboratory Standards Institute (CLSI) recommended procedure, Document M7-A7 (Matthew A.Wikler, 2009). Compounds have been evaluated against a panel of Gram-positive and Gram-negative organisms, including *Enterococcus faecium*, *Enterococcus faecalis*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Escherichia coli* and *Streptococcus pyogenes*. The MIC was determined as the lowest concentration of compound producing a >80 % reduction in fluorescence observed.
2.13. **hERG inhibition determination and cell cytotoxicity assays**

hERG Qpatch assay described in literature (Vasilyev et al., 2006). Cell cytotoxicity assays, described in literature (Crouch et al., 1993).

2.14. **Kinetic solubility assay**

Here, 5 mL of 10 mM DMSO stock solution was diluted to 100 mL with phosphate-buffered saline, pH 7.4, equilibrated for 1 h at room temperature, and filtered through Millipore Multiscreen HTS-PCF filter plates (MSSL BPC). The eluent was quantified by suitably calibrated flow injection CLND.

2.15. **Cytochrome P450 (CYP450)**

The P450 inhibition profile of the InhA inhibitors was determined as previously described in reference (Kajbaf et al., 2011).

2.16. **Tolerability studies**

For Tolerability studies C57BL/6 female mice of 18-20 g weight were used (n=6 mice). Drugs were administered as 1% methyl cellulose suspensions. The Up-and-Down method of experimentation is an adaptive procedure for conducting dose-response experiments having yes/no end-point. Animals are dosed one at a time at the best estimate MNLD. The dose for each successive animal is adjusted up or down, depending on the outcome of the previous animal. After reaching the reversal of the initial direction (the point where a decreasing dose pattern requires an increase due a tested animal’s survival or an increasing dose pattern results in a decrease due to lethality), four additional animals are dosed using the same up and down dose pattern. The maximal no lethal dose is calculated using the maximum likelihood algorithm. If any animal died or acute clinical signs were noted, the Up-&-Down procedure was carried out based on toxicological signs (functional observational battery). According to the Up-&-Down statistical procedure based on if, two dose levels were determined; a
dose that caused adverse clinical signs to 5% of animals (SD05) and a higher one that causes signs of toxicity at 95% of animals (SD95). Functional observational battery (FOB) and bodyweight gain were recorded and the animals were killed 24 hours and 48 hours after treatment and blood was sampled by intra-cardiac puncture for clinical chemistry and hematology analysis in He-Li tubes. Necropsy was carried out and the major organs were weighed and preserved in formalin.

2.17. Microsomal fraction stability experimental procedure

Pooled mouse and human liver microsomes were purchased from Xenotech. Microsomes (final protein concentration 0.5 mg/ml, 5 mM MgCl₂) and test compound (final substrate concentration 0.5 µM; final DMSO concentration 0.5 %) in 0.1 M phosphate buffer pH 7.4 were pre-incubated at 37°C prior to the addition of NADPH (final concentration 1 mM) to initiate the reaction. The final incubation volume was 600 µl. Control samples were included for each compound tested where 0.1 M phosphate buffer pH 7.4 was added instead of NADPH (minus NADPH). Midazolam was included as control in every experiment. Each compound was incubated for 30 minutes and samples (90 µl) were taken at 0, 5, 10, 20 and 30 minutes. The minus NADPH control was sampled at 0 and 30 minutes only. The reactions were stopped by the addition of 200 µl of acetonitrile:methanol (3:1) containing an internal standard, followed by centrifugation at 3700 rpm for 15 minutes at 4°C to precipitate the protein. Quantitative analysis was performed using specific LC-MS/MS conditions. Data analysis: from a plot of ln peak area ratio (compound peak area/internal standard peak area) against time, the gradient of the line was determined. Subsequently, half-life and intrinsic clearance were calculated using the equations below:

Elimination rate constant (k) = (- gradient)

Half life (t_{1/2}) (min) = \frac{0.693}{k}
Intrinsic Clearance (CLint) (ml/min/g tissue) = \frac{V}{0.693 \ t_{1/2}}

where V = Incubation volume ml/g microsomal protein and constant used for mg microsomal protein/gm liver is 52.5

2.18. Killing kinetics assay

Bacteria were grown at 37°C in 7H9 broth, 10% ADC, 0.025% Tyloxapol to mid-exponential phase and then diluted in 10 ml fresh Middlebrook 7H9 to an 5x10⁵ cfus/ml. Incubation was continued after the addition of compounds at 20xMIC. At specified time points, aliquots of cultures were withdrawn, serially diluted in 7H9 broth 0.025% Tyloxapol and plated on solid culture medium. Plates were then incubated at 37°C and CFU were counted after 3 to 4 weeks.

2.19. Timelapse imaging

Timelapse imaging of the GFP expressing MTB strain growing in the custom-made microfluidic device, was carried out as described before (Wakamoto et al., 2013). Briefly, cells were grown in 7H9 medium at 37 °C to mid-log phase and were seeded into the microfluidic device for imaging. Images were acquired at 1 h intervals on the phase and FITC (Excitation 490/20; Emission 528/38) channels using a DeltaVision personalDV imaging system (Applied Precision) equipped with a 100x objective (Olympus UPLFL 100x PH, 1.3 NA) and a CoolSnap HQ2 camera. Medium and compound were replenished every 24 h. As an endpoint assay, 1.0 μg/ml propidium iodide was added to the flow medium for 24h to stain cells with permeabilized cell envelopes and imaging done on the red channel (Excitation 555/28; Emission 617/73). Analysis of the time lapse image stacks was carried out using ImageJ v 1.47a (http://rsb.info.nih.gov/ij/). The survival curves depicted in Figure 4 were generated by tracking cell division and lysis events over the course of the experiment using the “Cell
Counter” plugin of ImageJ. Cytolysis was scored visually by abrupt loss of GFP fluorescence and abrupt decrease in phase intensity. Statistic analysis and fitting of data was performed using Prism (GraphPad).

2.20. Mice
Specific Pathogen-free 6-8-week-old female C57BL/6j mice (18 – 20g) are obtained from Harlan (Harlan Interfauna Iberica, Spain). The experiments were performed at AAALAC-accredited GlaxoSmithKline Laboratory Animal Science animal facilities in Tres Cantos (Madrid, Spain). The mice were kept in air-conditioned facilities with fifteen air changes per hour. Room temperature and relative humidity were 22 ± 3 ºC and 40- 70%, respectively. The mice were accommodated in groups of up to five individuals in Tecniplast® type IV cages with autoclaved dust free corncob bedding (Panlab, Barcelona, Spain). The mice were maintained under a twelve hours light/dark period. Autoclaved tap water and γ-irradiated pelleted diet were provided ad libitum.

2.21. Pharmacokinetic studies
Pharmacokinetic studies were performed in C57BL/6j female mice of 18-20 g weight (n = 4 mice per time point). Experimental compounds were administered by intravenous route at 0.5 (GSK613 and GSK625) or 4 mg/kg (GSK693) single dose at a volume of 10 ml/kg and by oral gavage at 50 (GSK613 AND 625), 100, 150 or 300 mg/kg single doses (GSK693) at a volume of 20 ml/kg. All mice received treatment in the fed state. Compounds were administered as solution in 1% DMSO, 7.5% PEG400, 20% Encapsine in saline for intravenous route and as 1 % methyl cellulose suspension for oral route. Whole blood was the compartment chosen for the establishment of compound concentrations: blood samples were taken by cardiac puncture for each mouse (euthanized by CO2) at 5, 15 and 30 minutes, 1, 1.5, 2, 3, 4 and 8 hours (n = 4 mice per time point) for intravenous route and at 15, 30 and 45 minutes, 1, 1.5, 2, 3, 4
and 8 hours (n = 4 mice per time point) for oral route. LC-MS was used as the analytical method of choice for the establishment of compound concentration in blood with a sensitivity of LLQ = 1-5 ng/ml in 25 µl blood. The non-compartmental data analysis (NCA) was performed with WinNonlin 5.2 (Pharsight, Certara L.P) and supplementary analysis was performed with GraphPad Prism 5 (GraphPad Software, Inc).

2.22. In vivo acute efficacy assessment
The experimental design has been previously described (Rullas et al., 2010). In brief, mice were intratracheally infected with 100,000 CFU/mouse (M. tuberculosis H37Rv). Products were administered for 8 consecutive days starting one day after infection. Lungs were harvested 24 hours after the last administration. For the chronic assay, mice were infected with 100 CFU/mouse and the products administered daily (7 days a week) for 8 consecutive weeks starting 6 weeks after infection. All lung lobes were aseptically removed 24 hours after the last administration, homogenized and frozen. Homogenates were plated in 10% OADC-7H11 medium for 14 days at 37ºC. Homogenates from compound treated mice were incubated for 18 days at 37ºC in plates supplemented with 0.4% (wt, vol) activated charcoal (Sigma Aldrich) to prevent the effect of product carryover. Moxifloxacin (Sequoia Research Products Ltd) was prepared as solution in 20% Captisol(R)/water.

2.23. In vivo chronic efficacy assessment
Specific pathogen-free, 8-10 week-old female C57BL/6j mice were purchased from Harlan Laboratories and were allowed to acclimate for one week. In brief, mice were intratracheally with 100 CFU/mouse and the products administered daily (7 days a week) for 4 or 8 consecutive weeks starting 6 weeks after infection. Lungs were harvested 24 hours after the last administration. All lung lobes were aseptically removed, homogenized and frozen. Homogenates were plated in 10% OADC-7H11
medium supplemented with Activated Charcoal (0.4%) for 18 days at 37°C. The positive control of isoniazid was assayed at 25 mg/kg, in distilled water.

3. Results

3.1. Biochemical, physicochemical and ADME characterization of hits GSK613 and GSK625

Several screening campaigns were run against InhA yielding an overall hit rate of 0.63% after quencher removal. The campaign covered a total of 1.9 million compounds unearthing GSK613 and GSK625 as the most attractive representative structures of the thiazoles series. Both compounds exhibited a single-digit nanomolar activity in the enzyme assay and micromolar in the whole cell assay and showed selective antibacterial activity against *M. tuberculosis*. The physicochemical properties and preliminary ADMET profile were completed (Table 1) and used to drive medicinal chemistry efforts.

N-(1-(2-chloro-6-fluorobenzyl)-1H-pyrazol-3-yl)-5-(1-(3-methyl-1H-pyrazol-1-yl)ethyl)-1,3,4-thiadiazol-2-amine (GSK613) was progressed to enzymatic mode of action studies to discard non-specific enzyme inhibition. GSK613 is a selective (inactive against human FAS), reversible inhibitor in addition to time, enzyme and solvent independent. Steady state competition experiments have shown that the hit competes with the fatty acid substrate for binding to the enzyme but is uncompetitive with NADH, consistent with inhibitors binding to a pre-formed enzyme:NADH complex (see Supplementary Fig. 1, Supplementary Table 2).

3.2. Use of InhA overexpressor strain to confirmation whole cell mode of action

Two *Mycobacterium smegmatis* mc²155 and *Mycobacterium bovis* BCG strains expressing increased levels of InhA protein from *M. smegmatis* and *M. tuberculosis* were constructed in order to identify inhibitors whose whole-cell activity is mediated
mainly or solely by inhibition of InhA (see Supplementary Fig. 2). A reproducible shift
in the Minimum Inhibitory Concentration (MIC) values higher than or equal to 4-fold in
strains overexpressing the target was considered as indicative that antitubercular activity
is mainly mediated by InhA inhibition. Thiadiazoles GSK625 and GSK613 met this
criterion.

3.3. Structure of the InhA:GSK625 complex
Crystal structures were obtained for InhA in complex with NAD⁺ and the thiadiazole
compound GSK625 at 2.9 Å resolution (see Supplementary Table 1) (PDB ID 5JFO).
The electronic density observed was used for identifying the main scaffold and its
disposition and interactions within the active site (Fig. 1).
GSK625 was observed bound to the hydrophobic substrate binding pocket of InhA,
which was surrounded by residues M199, L207, I215, M103, F149, Y158, A198 and
I202 (residue numbering as in reference (Lu et al., 2010; Rozwarski et al., 1999)). We
were able to crystallize the enzyme:inhibitor complex only in the presence of the
cofactor NAD(H), in agreement with the uncompetitive character of these inhibitors
relative to NADH (Fig. 1). When the structures of the InhA:GSK625 and that of the
InhA:NAD⁺:C16-fatty acyl substrate analog complex (Rozwarski et al., 1999) were
compared, it was observed that the aromatic pyrazole ring of the inhibitor occupied the
same pocket as the thioester and the trans double bond of the C16-substrate analog, in
close proximity to NAD⁺, which is in agreement with the competitive behavior of these
inhibitors relative to the DDCoA substrate.
The pyrazole ring interacted with NAD⁺ via π-π stacking (4 Å). In addition, the pyrazole
nitrogen H-bonded to the 2’-hydroxyl of the ribose moiety in the cofactor (3.3 Å). The
other key interactions identified were the direct H-bonding between (i) the nitrogen of
the thiadiazole ring and the amide NH of Met98, and (ii) the nitrogen linking the
pyrazole and the thiadiazole rings and the carbonyl oxygen of Met98 (2.9 & 2.5 Å, respectively). Hydrophobic and van der Waals interactions between the inhibitor and the side chains of the active site residues Met161, Phe97, Leu207, Met199, Met103, Gly104, Met98, Ala198, Gly96, Phe149, Tyr158 and Ile202 dominated the ligand-protein interactions. The halide substituted aryl ring of GSK625 interacted with the main chain Met199, and Gly104, through its halogen atoms, which increased the potency remarkably compared to the analogs without the aryl moiety. Noticeably, the catalytic residue Tyr158 did not make any H-bonds with the inhibitor and participated in ligand binding through van der Waals interactions only (3.9 Å). Thus, GSK625 is a representative of a class of inhibitors which does not need this conserved network of interaction with Tyr158 for potency. Furthermore, in the InhA:NAD⁺:GSK625 complex, the side chain of Phe149 adopted the identical position as in InhA:NAD(H) structure.

3.4. The thiadiazole series is bactericidal

To test if the these inhibitors shared the fast killing mode of action of INH, we determined the in vitro killing rates of GSK625 at 20xMIC in growth medium inoculated with approximately 10⁶ bacteria, during seven days (Fig. 2). After the first two days of incubation, the thiadiazole behaved similarly to INH, killing more than 99% of the initial bacteria. Due to the high frequency of spontaneous resistance to INH, the number of bacteria increases after 4 days of culture in presence of this drug, but not so in cultures treated with GSK625, where the number of colony forming units kept decreasing down to the detection limit.

3.5. GSK625 generates resistant mutants at lower frequency than INH and mutations map in the InhA active site

The in vitro rate of spontaneous resistance for GSK625 at 20xMIC was 3.7x10⁻⁸ mutants/c.f.u., very similar to the frequency obtained with rifampicin and three orders of
magnitude lower than that for isoniazid (Zhang and Yew, 2009). This rate is consistent with the presence of a single defined target. GSK625-resistant mutants exhibited a shift in MIC value by at least one order of magnitude and display no cross-resistance with other antitubercular compounds such as INH and rifampicin. Since the compounds bind to the enzyme competitively with DDCoA, it could be expected that mutations in the substrate-binding site could affect the interaction of the thiadiazoles, while leaving isoniazid (INH) and ethionamide (ETH) activity unaffected. The \( \text{inhA} \) alleles from single colonies of the seventeen mutants were amplified and sequenced. Four different point mutations were found in the coding regions from all mutants, producing a single amino acid change at either of two positions: Gly96 or Met103. These two amino acids map to the active site of the enzyme (see Supplementary Table 3) and have not been reported to be involved in resistance to InhA neither for isoniazid or ethionamide.

In order to link the point mutations in \( \text{inhA} \) to the resistant phenotype, the enoyl-ACP-reductase from the wild type and the four different point mutants were over-expressed and purified as untagged protein in \( E. \ coli \). All the mutated enzymes behaved similarly than the wild type and a very good correlation between enzymatic IC\(_{50}\) for inhibition with GSK625 and the MIC values of the source strains was observed (see Supplementary Table 4). For the whole-cell analysis, the complete \( \text{inhA} \) operon (\( \text{fabG1- inhA-hemZ} \)) from the wild type H37Rv strain and from the resistant mutants were cloned in a multicopy plasmid and transformed in fast growing \( M. \ smegmatis \) mc\(^2\)155 and in \( M. \ tuberculosisis \) H37Rv. In all cases, genetic transformation of the mutant operons conferred resistance to thiadiazoles well above that obtained by expressing the wild type version from the multicopy plasmid (see Supplementary Fig. 3).
3.6. Activity of GSK625 against sensitive and M(X)DR *M. tuberculosis* clinical isolates

InhA inhibitors, such as INH and ETH have been in use for decades and resistant MTB strains have emerged without identifiable mutations in *inhA* or in any other studied gene (Sandgren et al., 2009). It was therefore relevant to test a variety of clinical drug-resistant isolates for the possibility of pre-existing cross-resistance to the thiadiazole class. Two thiadiazole compounds were tested in a first instance against a group of INH-resistant clinical isolates with characterized mutations, either in *katG* (the most frequent resistant class) or in nucleotide position 15 upstream of the transcription start site for *inhA* (InhA over-expressor) (Sandgren et al., 2009; Vilcheze and Jacobs, Jr., 2007). The compounds retained full activity against the first group of mutants, while a moderate shift (4-8 times) in MIC was observed against the group of strains over-expressing InhA, as described above for laboratory constructs (Table 2).

GSK625 has been tested against recent clinical isolates of *M. tuberculosis* at two different TB reference centers (National Institute of Health Carlos III, Madrid and the Supranational Reference Laboratory of the Hospital Vall d’Hebron, Barcelona) and two observations were noteworthy. The first one was that the MIC\(_{90}\) obtained with 100 recently isolated MTB stains is 3 µM, showing that the thiadiazole sensitivity of our laboratory H37Rv strain is representative for the majority of MTB strains isolated in Spain, which are therefore sensitive to the compound (see Supplementary Table 5). The second was that GSK625 is active against most resistant isolates present in the sample, with MIC values similar to that of laboratory strain H37Rv independently of the resistance profile of the clinical strains (see Supplementary Table 6).
3.7. Lead optimization summary, lead profile

Despite the interesting *in vitro* anti-tubercular profile stated above, the initial hits were affected by a number of compound development liabilities such as physicochemical properties and DMPK profile. Further medicinal chemistry efforts (details to be reported elsewhere) were hence dedicated to the identification of optimized compounds. These efforts led to the identification of the chiral compound GSK693 as a promising lead (Table 3).

GSK693 retained the enzymatic activity of previous GSK613 and GSK625 hits. Additionally, GSK693 displayed equal potency against the *M. tuberculosis* H37Rv inside and outside of macrophages (0.2 µM). This was accompanied by a good CYP 3A4 inhibition profile. Early safety assessment was carried out, across different enzymatic assays. GSK693 did not show any sign of cytotoxicity and no hERG potassium channel inhibition (Vasilyev et al., 2006), suggesting a low risk for general toxicity and cardiotoxicity. Genotoxicity alert was discarded with a full Ames test (McCann et al., 1975). The breakthrough in the developability was obtained with the decreased lipophilicity, the enhanced solubility and the reduced metabolic liabilities whilst simultaneously achieving good mouse pharmacokinetic exposure levels after a single oral administration at different doses (see Supplementary Table 7). Cross resistance experiments with previously isolated mutants proved that the observed TB activity for the lead compound was target related (data not shown). Additional biological studies were carried out to confirm and gain further insight into the mode of action of GSK693.

3.8. Single cell analysis of effect of GSK693 on *M. tuberculosis*

To confirm that the synthetized derivatives retain the antitubercular properties of the original hits, we evaluated the bactericidal activity of GSK693 against MTB at the
single cell level by culturing the bacteria in microfluidic devices and observing their behaviour by time-lapse microscopy. MTB expressing GFP was grown in a microfluidic device for 6 days until they formed small microcolonies. The bacteria were then exposed to 5 µM (2.1 µg/ml- 25x MIC) GSK693 for 10 days, followed by washout of the drug for 7 days. Exposure to GSK693 induced a rapid growth arrest of individual MTB cells quite similar to the response of MTB when exposed to INH. Interestingly, while in case of INH, the cells continued to divide without elongation for several hours and started to lyse after a lag of several days, in case of GSK693, the cell lysis was more rapid without any significant lag period (see supplementary Fig. 4). While the total fraction of cells undergoing lysis was comparable between GSK693 (53±20% N=32 microcolonies) and INH (42±15%, N=41 microcolonies), the bacteriolysis induced by GSK693 was significantly more rapid than INH (P=0.011, Log-rank Mantel-Cox test). In both cases, after exposure to INH or GSK693, the rate of killing was maintained even after washout of the drug, indicative of strong post-antibiotic effect or long-term inhibition of the target by the compounds. In order to identify cells that did not lyse visibly but whose cell wall was potentially compromised, we stained the remaining intact cells with propidium iodide (PI), as an endpoint assay. About 22% of the cells still intact after GSK693 exposure were found to be PI-positive and remarkably the remaining 78% of the cells were still able to exclude PI. None of the intact bacteria exhibited regrowth after washout of the drug, possibly suggestive again of long term target inhibition or possible pushing of the surviving bacterial population into a non-replicative senescent state.

3.9. Confirmation of KatG-independent activity of GSK693

As shown previously in Table 2, thiadiazole compounds exhibited no change in MIC against INH-resistant clinical isolates carrying mutations in katG. However, since MIC
is more of an assay of bacteriostatic activity we decided to confirm the *katG*-independent bactericidal activity of GSK693. We compared the killing efficacy of INH vs GSK693 against a panel of three strains differing in the level of KatG expression – (i) wild type *M. tuberculosis* with the native *katG* locus (ii) a *katG* null mutant of *M. tuberculosis*, deficient in catalase activity (Ng et al., 2004) and (iii) a *katG* overexpressing strain of *M. tuberculosis*, that carries a second copy of MTB *katG* at the atTB locus. These strains were exposed to 3.6 μM (0.5 μg/ml) INH or 5 μM of GSK693 for 4 days and the fraction of bacteria surviving antibiotic treatment determined by plating. As expected, against INH the *katG*-null mutant was resistant and the *katG*-overexpressing strain was more sensitive compared to the wild type strain (Fig. 3). Encouragingly and consistent with our previous observations, the *katG*-null mutant strain was killed equally well as the wild type strain by GSK693. This further reinforces the *katG*-independent nature of bactericidal activity of GSK693.

3.10. *in vivo* efficacy of GSK693 in the acute and chronic TB murine infection models

Given its desirable and balanced profile, GSK693 fulfilled our requirements to be progressed to tolerability and *in vivo* efficacy studies in both acute and chronic TB murine infection models. The maximal no lethal dose (MNLD) in mice was found to be higher than 1000 mg/Kg. Subsequently, the oral efficacy of GSK693 was evaluated in an acute murine model as previously described (Rullas et al., 2010) at doses of 30, 100 and 300 mg/Kg u.i.d. for 7 days, using Moxifloxacin as a positive control at 30 mg/Kg. The compound showed a clear dose/response pharmacological effect with this dose range (Fig. 4a). In mice treated with 100 mg/kg, the bacterial load was reduced 4 log10 colony forming units (CFU) per lung, matching the reduction obtained with 30 mg/Kg of Moxifloxacin.
The important proof-of-concept in vivo efficacy was obtained by GSK693 in the acute murine TB model. GSK693 was further evaluated in an established model of TB infection at a dose of 300 mg/kg as suspension in 1% methylcellulose. After two months treatment of chronically infected mice, GSK693 (300 mg/Kg) and Isoniazid (25 mg/Kg), showed a clear efficacy (Fig. 4b) and the differences observed between both groups were not statistically significant.

The bacterial load reduction in both acute and chronic murine models with GSK693 at 300 mg/Kg is virtually the same than the one observed with isoniazid at the human equivalent dose.

4. Discussion

Isoniazid (INH) is together with rifampicin the corner stone of the TB treatment, it has been administrated to more than 40 million people saving millions of lives. Its outstanding cidal effect rapidly blocks bacterial spread and significantly improves patient’s conditions after a few weeks of treatment.

On the basis of INH mode of action, it has been proposed that direct inhibitors of the enoyl-ACP-reductase of Mtb can retain the outstanding antitubercular profile of INH overcoming most of the issues associated with the drug, such us drug resistance and toxicological effects. Additionally, compounds inhibiting InhA without requiring activation by KatG could be active under anaerobic conditions (Vilcheze et al., 2011), where catalase-mediated activation is suppressed by the lack of oxygen (Karakousis et al., 2008).

Following the above mentioned rational, a biochemical assay based on NADH consumption in the presence of docecenoyl-CoA by purified InhA enzyme has allowed the identification of different direct inhibitors of Mtb enoyl-ACP reductase. All the compounds showing IC$_{50}$ values in the micromolar range were clustered and tested for
antitubercular activity against wild type and InhA overexpressor strains to identify five chemical series of bona-fide inhibitors (whole cell activity clearly linked to enzymatic inhibition).

Enzymatic and whole cell activities will provide the desired efficacy at an acceptable dose, but a drug also needs to have the right physicochemical properties to increase the probability of success (Hann and Keseru, 2012). The drug-like chemical properties of the selected InhA inhibitors were evaluated to select thiadiazoles series as the family with the best balance between potency and physicochemical properties.

GSK613 and GSK625 are the most attractive representative compounds of the thiadiazoles series. Both exhibited a single-digit nanomolar activity in the enzyme assay and micromolar in the whole cell assay and showed a selective antibacterial activity against *M. tuberculosis*. The series does not required KatG activation and retain the antitubercular activity against sensitive, MDR and XDR clinical isolates.

The in vitro frequency of spontaneous resistance is three orders of magnitude better than the one obtained for isoniazid. The four different point mutations identified conferring resistance were found in *inhA* coding region, producing a single amino acid change at either of two positions: Gly96 or Met103. These two amino acids map to the active site of the enzyme and have not been reported to be involved in resistance to InhA neither for isoniazid or ethionamide.

Like other InhA direct inhibitors, thiadiazoles bind to the enzyme-NADH complex, but in contrast to previously crystallized InhA inhibitors such as pyrrolidine carboxamides (He et al., 2006), Genzyme10850 and triclosan (Kuo et al., 2003), thiadiazoles do not establish any direct interaction with Tyr158, and unlike the isoniazid-NADH adduct,
GSK625 does not cause the flipping of the Phe149 side chain and thus there is no interaction with the isonicotinic acid binding pocket.

Recently, it was proposed that high affinity slow binding inhibition of InhA is related to the ordering of the substrate binding loop, brought about by the long residence time of inhibitors on the enzyme (Lu et al., 2010). Importantly, in all of the co-crystal structures with thiadiazoles the substrate binding loop (residues 197-226) was ordered and well resolved in the crystal structure, as previously seen with high-affinity slow onset FabI inhibitor structures, implying that the compounds may also be slow, tight binding inhibitors, which generally enhances the \textit{in vivo} activity and increases their potential as antitubercular drugs.

The medicinal chemistry efforts have been focused on the improvement of the physiochemical properties and DMK profile. GSK693 retains all the antitubercular properties shown by the initial hits accompanied by a good cytochrome P450 profile, lack of cardiovascular liabilities, non-mutagenic, reduced lipophilicity and good solubility which translate to good oral bioavailability. Dosed orally as a 1% methylcellulose suspension the compound has shown a clear antitubercular activity in the acute and chronic mouse models at free compound exposures similar to those obtained with isoniazid.

\section{5. Conclusion}

Standard antitubercular treatment consists of a combination of four compounds (isoniazid, rifampicin, ethambutol and pyrazinamide) during 2 months that eliminate all the fast growing bacteria and 4 additional months of isoniazid and rifampicin that complete patient cure.
The treatment is long, complex and has important adverse effects, so lack of adherence is not unusual, leading to suboptimal responses (failure and relapse), emergence of resistance, and continuous spread of the disease. The presence of rapidly bactericidal compounds like isonizid as part of the combination has a high impact in blocking transmission and improving the health of infected people.

We have demonstrated that a direct inhibitor on InhA can retain the outstanding profile of isonizid and considering the DMPK parameters of both compounds the antitubercular effect is achieved at similar exposures of free drug proving our initial hypothesis and overcoming resistances mediated by KatG. GSK693 has demonstrated in vivo efficacy comparable to the marketed drug Isoniazid without being a pro-drug, thus overcoming most of INH associated liabilities. Besides, its improved property profile should translate into a safer treatment. Overall, this achievement opens the door to the development of a direct inhibitor of the enoyl-ACP reductase (InhA) as an attractive drug candidate for the therapy of Tuberculosis.

**Author Contributions**

MMH and EPH have performed all the target-engagement experiments (whole cell mode of action tools development, mutant selection and characterization). DB performed MIC studies. SM has expressed and purified InhA mutated proteins for biochemical experiments. DAG has performed the biochemical experiments to support SAR of thiadizole series and mutant characterization. EA managed the screening campaign that identified the new series of inhibitors. MSJ and NMC determined the activity of the new compounds against sensitive and resistant clinical isolates. PT and PC performed in vitro and in vivo cytotoxicity studies respectively. AGP, FA and SH performed the PK bioanalysis under the supervision of SFB. JR performed in vivo studies directed by IAB. JLL performed molecular modeling and data analysis. GC
expressed and purified protein for structural experiments, obtaining and analyzing crystallographic data under the supervision of JCS. JCP directed chemistry. LE, SGV, RFM and LLB synthesized compounds and participate in data analysis and compound designed. ND, FSC and JDM generated single cell microscopy data. DB organized and coordinated ORCHID consortium. JFGB, EPH and AML organized and planned biology studies. AML wrote the manuscript with input from the co-authors.
In memoriam statement

In memoriam of Emilio Alvarez, an example of professionalism, comradeship and inspiration. His scientific knowledge, advice and wisdom will be greatly missed.
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Table legends

Table 1. Structure of hit compounds GSK613 and GSK625. Compounds were assessed for activity against purified InhA, *M. tuberculosis* H37Rv, a panel of Gram positive and negative bacteria, and HepG2 mammalian cells, ClogP, and DMPK parameters (*in vitro* and *in vivo* clearance, volume of distribution at steady state (Vss), half-life ($t_{1/2}$), Tmax, Cmax and dose normalized exposure (DNAUC) in mice.

Table 2. Activity of the compounds against resistant clinical isolates.

Table 3. Structure and properties of optimized lead GSK693. The lead was assessed for activity against *M. tuberculosis* H37Rv both intra and extracellularly, physicochemical and ADMET properties.
Figure Legends

Figure 1. X-Ray structure of InhA-GSK625 complex: Binding mode of GSK625 to the InhA active site. GSK625 (green) interacted with NAD+ (gray) and M98 via H-bonds (shown as black dashed lines); with G96, F97, M98, M103, F149, M161, I202, G104, M199, and L207 through hydrophobic and van der Waals interactions. The two residues involved in resistance, G96 and M103, are colored in magenta. Atom coloring is: oxygen red, nitrogen blue, sulfur yellow, chlorine green and fluoride light blue.

Figure 2. Killing curve of GSK625. Killing rates of M. tuberculosis, exposed to 20xMIC of GSK625, INH and linezolid.

Figure 3. GSK693 exhibits KatG-independent bactericidal activity. This plot depicts the percent survival of wild type M. tuberculosis (WT); a katG-null catalase deficient mutant strain of M. tuberculosis (ΔkatG); and a katG-overexpressing strain of M. tuberculosis (katG-2X) after exposure to 3.6 µM INH or 5 µM GSK693 for a period of 4 days. Data shown represents the Mean ± SE of three independent replicates.

Figure 4. (a) Dose-response studies in acute murine model of TB efficacy. Log cfu in mice after oral treatment with different doses of GSK693 (30, 100 and 300 mg/Kg) and moxifloxacin at 30 mg/Kg. Treatment in all groups resulted in a significant clearance of bacilli compared with the controls (P < 0.001).

(b) Efficacy response of GSK693 in chronic, established murine model of TB infection. Log cfu in mice after oral treatment with GSK693 at 300 mg/Kg and INH at 25 mg/Kg. Treatment in both groups resulted in a significant clearance of bacilli compared with the controls (P < 0.001).
**Table 1**

|                       | GSK613 | GSK625 |
|-----------------------|--------|--------|
| InhA IC50             | 7 nM   | 2 nM   |
| *Mtb* MIC             | ≤1 µM  | 1 µM   |
| Antibacterial panel   | ≥64 µg/mL | ≥64 µg/mL |
| MW                    | 403.9  | 417.9  |
| ClogP                 | 3.75   | 4.05   |
| Art. Memb. Permeability | 3.4 · 10⁻⁵ cm/sec | 5.4 · 10⁻⁵ cm/sec |
| Solubility            | CLND   | 10 µM  | 9 µM  |
| HepG2 Cytotoxicity    | > 100 µM | > 100 µM |
| Tox 50                |        |        |
| In vitro Cli mouse    | 11.9 mL/min.g | 14.9 mL/min.g |
| In vitro Cli human    | 2.9 mL/min.g | 2.8 mL/min.g |

**In vivo pharmacokinetic studies in mice**

|                       |                      |                      |
|-----------------------|----------------------|----------------------|
| In vivo CI (0.5 mg/kg iv) | 156.7 mL/min/Kg     | 119.9 mL/min/Kg     |
| In vivo Vss (0.5 mg/kg iv) | 2.5 L/kg           | 1.5 L/kg           |
| In vivo t1/2 (0.5 mg/kg iv) | 0.3 h              | 0.2 h              |
| In vivo Tmax (50 mg/kg po) | 0.75 h             | 1 h                 |
| In vivo Cmax (50 mg/kg po) | 94.3 ng/ml         | 2383 ng/ml         |
| DNAUC (ng·h/ml per mg/Kg) | 5                   | 123                 |
Table 2

| Compound/Strain | Wild Type | -15 C/T inhA | katG S315T |
|-----------------|-----------|--------------|-------------|
|                 | H37Rv     | CI1 | CI2 | CI3 | CI4 | CI5 | CI6 | CI7 |
| GSK625          | MIC (µM)  | 1   | 8   | 16  | 8   | 2   | 1   | 2   | 2   |
| Ratio           | 1         | 8   | 16  | 8   | 2   | 1   | 2   | 2   |
| INH             | MIC (µg/ml) | 0.5 | 3.125 | 1.6 | 1.6 | 50  | 50  | 25  | 200 |
| Ratio           | 1         | 6   | 3   | 3   | 100 | 100 | 50  | 400 |
Table 3

| Physchem properties | Value |
|---------------------|-------|
| MW                  | 419.6 |
| ClogP               | 0.71  |
| Art. Memb. Permeability | $1.9 \cdot 10^{-5}$ cm/sec |
| Solubility CLND     | 413 µM |

| Activity profile   | Value |
|--------------------|-------|
| InhA IC$_{50}$     | 7 nM  |
| Mtb MIC            | 0.2 µM |
| Mtb intracell MIC  | 0.2 µM |

| cytochrome P450 profile | Value |
|-------------------------|-------|
| CYP 3A4 IC$_{50}$ VR    | > 50.1 µM |
| CYP 3A4 IC$_{50}$ VG    | 25.1 µM |

| Cytotoxicity profile   | Value |
|------------------------|-------|
| HepG2 Cytotoxicity Tox 50 | >50 µM |
| Cell Health (Memb.; Nucleus; Mitoch.) | >199.5 uM |

| Genetic Toxicity | Value |
|------------------|-------|
| Ames test        | Negative |

| Cardiovascular profile | Value |
|------------------------|-------|
| Qpatch IC$_{50}$       | >50 uM |

| In vitro Metabolic stability | Value |
|-------------------------------|-------|
| Cli mouse                     | 2.1 mL/min.g |
| Cli human                     | 0.2 mL/min.g |

| In vivo pharmacokinetic study in mice | Value |
|--------------------------------------|-------|
| Cl (4 mg/kg iv)                      | 83 mL/min/Kg |
| Vss (4 mg/kg iv)                     | 2.58 L/Kg |
| t1/2 (4 mg/kg iv)                    | 0.94 h  |
| Tmax (100 mg/kg po)                  | 0.42 h  |
| Cmax (100 mg/kg po)                  | 37271 ng/mL |
| DNAUC                               | 935.8 ng·h/ml per mg/Kg |
Figure 1

[Diagram showing molecular structures with labels such as Angle 1, Angle 2, NAD\(^+\), GSK625, M98, M99, G96, M103, G104.]
Figure 2
Figure 3
Figure 4a
Figure 4b

The graph shows the CFU/mouse (lungs) for different treatments: Pretreatment, Untreated, INH, and GSK693. The y-axis represents the CFU/mouse ranging from $10^0$ to $10^8$. The x-axis represents the different treatments. The inoculum and limit of detection are indicated by dashed lines.

* indicates significant differences.
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Conflict of Interest

All the authors confirm the absence of any conflict of interest.
HIGHLIGHTS

- KatG independent InhA inhibitors mimicking Isoniazid cidality
- GSK693 first DI showing oral *in vivo* efficacy similar to Isoniazid
- Retain activity against M(X)DR clinical isolates
- Physicochemical properties akin to antitubercular drugs