SUPPORTING INFORMATION (SI)

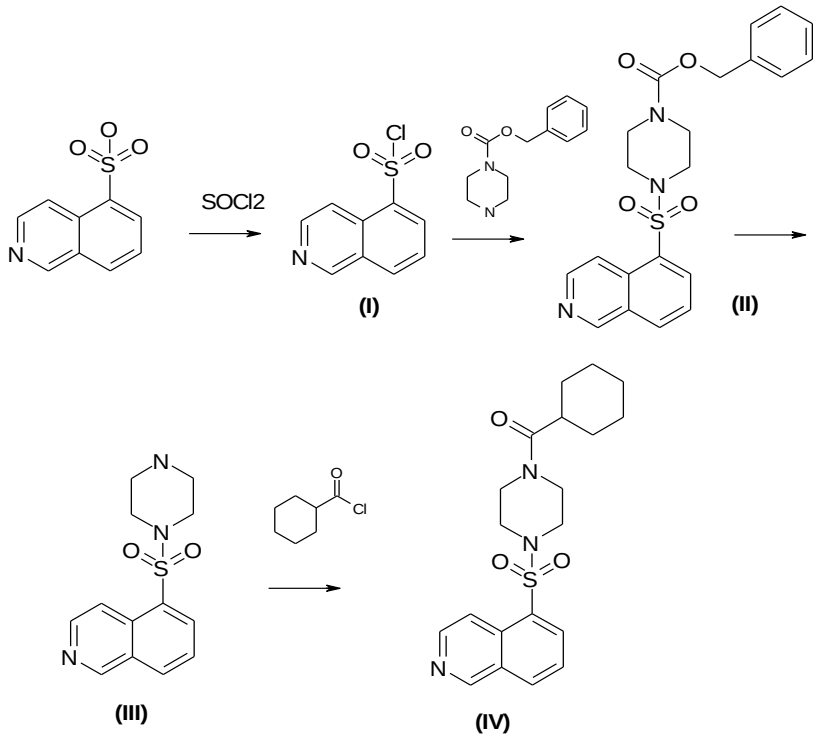
The inosine monophosphate dehydrogenase, GuaB2, is a vulnerable and bactericidal new drug target for tuberculosis

Vinayak Singh, Stefano Donini, Angela Pacitto, Claudia Sala, Ruben C. Hartkoorn, Neeraj Dhar, Gyorgy Keri, David B. Ascher, Guillaume Mondésert, Anthony Vocat, Andréanne Lupien, Raphael Sommer, Hélène Vermet, Sophie Lagrange, Joe Buechler, Digby F. Warner, John D. McKinney, Janos Pato, Stewart T. Cole, Tom L. Blundell, Menico Rizzi & Valerie Mizrahi

METHODS

Synthesis of VCC234718

The four-step synthesis of VCC234718 **(IV)** was carried out as follows.



1. *Isoquinoline-5-sulfonylchloride hydrochloride* (**I**)

5 g Isoquinoline-5-sulfonic acid was added in portions with stirring to 100 mL thionylchloride containing 1 mL of dimethylformanide. The reaction mixture was refluxed for 4 h while the starting material dissolved. After cooling to room temperature, thionylchloride was removed under vacuum, and the residue triturated several times with diethyl ether, yielding 16 g product. Purity was checked by TLC (eluent ethyl acetate) and the compound used in the next step without further purification.

2. *N-(5-Isoquinolinylsulfonyl)- N'-carbobenzyloxy-piperazine* (**II**)

8.3 g (**I**) and 5.5 g N-carbobenzyloxy piperazine were added to the heterogeneous mixture of 60 mL ethyl acetate and 50 mL saturated bicarbonate solution with vigorous stirring. After 12 h, the phases were separated, and the organic phase was washed with brine, dried on sodium sulfate, and then evaporated to dryness. 13.5 g yellow oil was obtained. LCMS: 92 %

3. *N-(5-Isoquinolinylsulfonyl)piperazine hydrochloride* (**III**)

13.1 g (**II)** was refluxed in 200 mL of 2 N HCl with stirring for 5 h. After cooling to ambient temperature it was extracted twice with diethyl ether then neutralized with concentrated NaOH solution and extracted again three times with ethyl acetate. The organic phase was separated, dried on sodium sulfate, and then ethyl acetate saturated with hydrochloric acid was added carefully. The hydrochloride salt of the product was crystallized and isolated by filtration. The solid was washed twice with ethyl acetate and once with diethyl ether, and then dried under vacuum. Yield: 7.4g. LCMS 99%.

H-NMR (d_6DMSO): 95%

9.73 (s, 1H); 9.40 (br. s, 2H); 8.77 (d, J = 6.4 Hz, 1H); 8.69 (dm, J = 8.0 Hz, 1H); 8.59 (dm, J = 6.4 Hz, 1H); 8.53 (dm, J = 7.5 Hz, 1H); 8.02 (dd, J = 8.0 and 7.5 Hz, 1H); 3.37 (m, 4H); 3.13 (m, 4H)

4. *N-(5-Isoquinolinylsulfonyl)- N'-cyclohexanoyl-piperazine* (**IV**)

0.2 g (**III**) and 140 µl cyclohexanecarbonylchloride were added to the heterogeneous mixture of 15 mL ethyl acetate and 15 mL saturated bicarbonate solution with vigorous stirring. After 12 h the phases were separated, the organic phase was washed with brine, dried on sodium sulfate, and then evaporated to dryness. The crude product was purified on silica column (eluent- chloroform : methanol, 10:1) yielding 150 mg of pure product. LCMS: 95%

H-NM (d_6DMSO): 100%

9.50 (s, 1H); 8.70 (d, J = 6.2 Hz, 1H); 8.52 (dm, J = 8.0 Hz, 1H); 8.44 (dm, J = 6.2 Hz, 1H); 8.36 (dm, J = 7.5 Hz, 1H); 7.88 (dd, J = 8.0 and 7.5 Hz, 1H); 3.50 (m, 4H); 3.07 (m, 4H); 2.46 (m, 1H); 1.44-1.69 (ovl. m, 5H); 1.00 (1.30 (ovl. m, 5H).

ADMET

Determination of the intrinsic clearance for VCC234718 using human hepatocytes: contribution of CYP3A4

The objectives of this study were to determine the *in vitro* metabolic hepatic intrinsic clearance and the contribution of CYP3A to the in vitro metabolic intrinsic clearance of VCC234718 using cryopreserved human hepatocytes in primary culture.

Incubation and experimental conditions with two lots of cryopreserved human hepatocytes (purchased from Celsis/In Vitro Technologies and KaLy-Cell) were as follows: cryopreserved hepatocytes 16 x 10^4 cells/well, collagen coated 48-well plates, incubation volume 100 µL; incubation medium, serum-free hepatocyte culture medium; VCC234718 concentration, 5 µM; sampling times, 0, 1, 2, 3, 4, 6 and 24 hours. Ketoconazole at a final concentration of 3 µM was used for the specific and potent inhibition of enzyme reactions catalyzed by CYP3A4. Quantification of VCC234718 in pooled supernatant and cells was performed using an electrospray UPLC-MS/MS method after the protein precipitation step.

The intrinsic clearance value was calculated according to the following equation: $Cl_{int\ in\ vitro} = k_e \cdot V$ (where $Cl_{int\ in\ vitro}$, is the *in vitro* intrinsic clearance expressed in $mL.h^{-1}.10^{-6}$ hepatocytes (or cells); k_e is the elimination rate constant expressed in h^{-1} calculated with WinNonLin, via a compartmental analysis, modelling an intravenous bolus injection with no lag time (first order elimination); V is the incubation volume expressed in mL normalized to 10^6 hepatocytes). Elimination rate constants were determined over 24 h in the uninhibited (K_e) and inhibited (K_{ei}) states. The relative CYP contribution to the overall Cl_{int} (f_m %) was determined using the following equation:

$$f_m\ (\%) = (1 - \frac{K_{ei}}{K_e})\ x\ 100$$

Metabolic stability assay

The metabolic lability was evaluated at a concentration of 5 µM in mouse, rat and human liver microsomes (human prepared a pool of 150 donors; animal prepared from Swiss CD1 mouse and SD rat); a time course was performed at 0, 5, 10, 20 & 30 min with NAPDH (1 mM) as cofactor. Quantification of unchanged compound was done by LC/MS-MS and results were expressed as *in vitro* intrinsic clearance in µL/min/mg protein. Intrinsic clearance values were calculated as follows: percentage of drug remaining is calculated for each compound by normalizing the data at different time point to T0 for analyte / internal standard peak area ratio: % stability = (peak area ratio at Tx / peak area ratio at T0); elimination slope (-k) was determined using a linear regression of the neperian logarithm (ln) of the percent remaining drug versus incubation time (k is expressed in µM / minute). Intrinsic clearance (Clint) calculation is µL/min/mg: Clint = k * incub volume / protein quantity in incub * substrate initial concentration (Incubation volume is expressed in µL; Protein quantity in incubation is expressed in mg; Substrate initial concentration is expressed in µM). If k is

expressed in min^{-1} (coming from Ln (% stab) plotted against time), intrinsic clearance (Clint) calculation is µL/min/mg: Clint = k / protein concentration in incub.

hERG assay

Pharmacological activity of the compounds at cardiac hERG channels (Kv11.1) was assessed functionally using an automated planar patch-clamp electrophysiology station. In brief, CHO cells stably expressing human hERG channels were grown under the permanent pressure of the appropriate selection antibiotics according to the specifications of the manufacturer (hERG-Duo cells, B'SYS GmbH, Switzerland). Cells were harvested below 80% confluence, suspended in serum-free medium and dispensed automatically to multi-well recording plates proprietary to a QPatch HTX workstation for whole-cell voltage-clamp recordings (Sophion, Biolin Scientific AB, Denmark). The composition of the extracellular recording buffer was (in mM): NaCl, 145 ; KCl, 4 ; $CaCl_2$, 2 ; $MgCl_2$, 1 ; HEPES, 10 and glucose, 10, adjusted to pH 7.4 with NaOH. The intracellular buffer contained (in mM): KCl, 120 ; $CaCl_2$, 5.37 ; $MgCl_2$, 1.75 ; EGTA/KOH, 10/31 ; HEPES, 10 and Mg-ATP, 4, adjusted to pH 7.2 with KOH. The voltage protocol used to activate hERG currents consisted of a first depolarizing step from a -80mV holding potential to +20 mV for 5 s. Then, a second repolarizing step to -50 mV was applied for another 5 s, followed by return to holding at -80 mV. Cycle time was 15 s, recordings were performed at room temperature and drug effects were evaluated on the peak tail-current elicited by the second repolarizing step. For pharmacology, stock solutions of the test articles at 10 mM were prepared in DMSO and delivered frozen by Sanofi's internal compound management services. A 6-point concentration–range was prepared in polypropylene micro-titer plates in two steps by first serially diluting the initial 10 mM stock 1:3 in DMSO and then diluting each of the 6 intermediate working solutions 1:333 in extracellular recording medium containing 0.06% Pluronic F-68 (Gibco/Thermofischer, France). Final concentrations of test articles (i.e. 0.12 µM, 0.37 µM, 1.1 µM, 3.3 µM, 10 µM

and 30 µM) were applied cumulatively by the pipetting arm of the QPatch workstation in ascending order for 2.5 min each. A positive control (Terfenadine, 10 µM) was added at the end of each recording sequence. Time-course of hERG tail current amplitude was analyzed off-line following optional leak current subtraction and compensation of current rundown as needed. Half-maximal inhibitory concentrations (IC_{50}) were obtained from at least 3 cells by fitting current amplitudes measured at the end of each exposure period and normalized with respect to pre-drug baseline with a four parameter sigmoidal Hill equation.

***In vitro* study of the trans-epithelial intestinal transport of VCC234718 using Caco-2/TC-7 cell monolayers**

The TC7 clone was established from the parental Caco-2 cell line, a human colon adeno-carcinoma cell line, and was obtained from INSERM U-178 (Pr A. Zweibaum, Villejuif, France). They were maintained and sub-cultured according to the procedure in use. For transport experiments, Caco-2/TC7 cells were seeded on Transwell® clear inserts in Falcon HTS 96 square well plates and were used between day 15 and day 25 of culture.

The permeability coefficient was determined under standard conditions: Apical compartment: HBSS; pH 6.5; 0.5 % BSA; Basal compartment: HBSS; pH 7.4; 5 % BSA; Test compound concentration: addition of 20 µM final VCC234178 in the Apical chamber. The “Apical to Basal” transport of VCC234718 was measured by a sampling aliquot from the Basal chamber at t = 120 min. The recovery of VCC234718 was determined at the end of the incubation period. Samples were quantified by LC-MS/MS. Cimetidine (20 µM) was used as a low reference compound. Recovery was calculated according to the following equation: % recovery = ((A2 + B2) / A0) × 100 where A0 was the amount of VCC134718 in the apical compartment at the beginning of incubation, A2 and B2 the amounts of VCC134718 in the apical and basal compartments at the end of incubation, respectively.

The permeability coefficient was calculated according to the following equation: Ptot = B2 / (A0 × S × T) where B2 was the amount of VCC134718 in the basal compartment at the end of incubation, A0 the concentration of VCC134718 in the apical compartment at the beginning of incubation, S the filter area (cm^2) and T the incubation time (s).

Bacterial culture conditions

Mtb strains were grown in Middlebrook 7H9 media (Difco) supplemented with 0.2 % glycerol, Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco) and 0.05 % Tween 80 or on solid Middlebrook 7H10 media (Difco) supplemented with 0.5 % glycerol and OADC. Hygromycin (Hyg), kanamycin (Km) and gentamycin (Gm) were used in mycobacterial cultures at final concentrations of 50, 25 and 2.5 µg/mL, respectively. ATc (Sigma) was used at concentrations up to 500 ng/mL. Conditional mutants in the Tet-ON and Tet-OFF configurations were cultured, and transcriptional silencing carried out, as previously described.[1-2]

Drug susceptibility testing

The Alamar Blue (AB) assay was used for MIC testing by broth microdilution, as described previously.[1-2] The time-kill kinetics of *Mtb* H37Rv exposed to VCC234718 over a drug concentration range of 0-32 µM were assessed as previously described.[1] Serial dilutions of each sample were plated on 7H10 agar at days 0, 1, 2, 3, 5 and 7 and CFUs were scored following incubation at 37°C for 3-4 weeks. The streptomycin-dependent *Mtb* 18b strain and its luminescent derivative, 18b-Lux, were used to assess the activity of VCC234178 against non-replicating bacilli, as previously described. [3-4] The intracellular activity of VCC718 was assessed in MRC-5 fibroblasts and activated THP-1 macrophages by monitoring the ability of the compound to protect the cells from *Mtb*-induced lysis, as described by Rybniker *et al.* [5]

Isolation and characterization of VCC234718-resistant mutants of *Mtb*

Cultures of wild type *Mtb* were grown at 37°C to an OD_{600} = 0.8, pelleted by centrifugation, and resuspended in Middlebrook 7H9 broth supplemented with glycerol, OADC, and 0.05% Tween 80. Aliquots containing 10^8 or 10^9 cells were plated on Middlebrook 7H10 agar supplemented with glycerol and OADC in the presence of VCC234718 at 10×, 20×, and 50× the MIC_{90} value determined in liquid culture. Colonies arising after 4 weeks' incubation were picked and sub-cultured in Middlebrook 7H9 broth and the resistance phenotype confirmed by MIC testing.

Construction of mutant strains of *Mtb* with altered expression of GuaB2

To construct *Mtb* strains carrying an integrated copy of *guaB2* or *guaB2*^{Y487C}, the plasmids pTTguaB2 and pTTGuaB2^{Y487C} carrying *guaB2* or *guaB2*^{Y487C} under control of the native promoter of the *guaB2-guaB3* operon were constructed by PCR amplification of the 1920 bp region spanning the *guaB2* ORF and 330 bp of upstream sequence using genomic DNA from H37Rv or the VCC234718-resistant strain, SRMV2.6, as template, and the primers listed in Table S5. To construct *Mtb* strains carrying an integrated copy of the wild type *guaB2-guaB3* operon, a 3086 bp region spanning the *guaB2-guaB3* genes and 330 bp of upstream sequence was amplified using genomic DNA from H37Rv and the primers listed in Table S6. The amplified products were digested with *Eco*RI and cloned in the Tweety-based integrating plasmid pTT1B::Gm.[2] The resulting plasmids, pTTguaB2, pTTguaB2^{Y487C} and pTTguaB2-guaB3 were electroporated individually into H37Rv or SRMV2.6 to produce the strains, H37Rv *attB*::*guaB2*, H37Rv *attB*::*guaB2*^{Y487C}, H37Rv *attB*::*guaB2-guaB3*, SRMV2.6 *attB*::*guaB2*, SRMV2.6 *attB*::*guaB2*^{Y487C}, and SRMV2.6 *attB*::*guaB2-guaB3*, respectively.

To construct a mutant of *Mtb* in which the *guaB2-guaB3* promoter was replaced by the Tet-regulated promoter, $p_{myc1}tetO$[6], a suicide plasmid carrying an amplicon spanning the RBS and 707-bp 5'-terminal region of *guaB2*, cloned as a *Sph*I/*Not*I fragment in pSE100[7] was electroporated into *Mtb* H37Rv and transformants selected on Middlebrook 7H10 agar supplemented with OADC and Hyg. Individual colonies were grown to mid-logarithmic phase in Middlebrook 7H9 broth supplemented with OADC and Hyg. The site-specificity of homologous recombination in the putative single-cross-over (SCO) recombinants was confirmed by Southern hybridisation using the ECL™ Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences). Modulation of *guaB2* expression using ATc was achieved by electroporation of pGMCK-OX21-T10 and pGMCK-OX38-T28[8] into the SCO recombinant, *guaB2-guaB3*-SCO, to generate conditional mutants in the Tet-ON_M and Tet-OFF configurations, respectively. Integration into the chromosome was facilitated by co-delivery of an additional suicide vector, pGA-OXP15-intL5, transiently expressing integrase.[8] The transformants were selected on Middlebrook 7H10 supplemented with OADC, Hyg and Km, in the presence and absence of ATc (200 ng/mL). The vector, pTTguaB3, was used to complement the *guaB2-guaB3* Tet-OFF strain with *guaB3* expressed under the control of native promoter. To construct pTTguaB3, PCR amplification of the *guaB2-guaB3* operon, with an in-frame deletion of the *guaB2* gene, was performed by the splice-by-overlap-extension method.[9] Briefly, a first-round of PCR amplification of fragments flanking *guaB2* was performed using primers Rv3012c-EcoRI-For/ guaB2'-SOE-Rev, and guaB3-SOE-For/ guaB3-EcoRI-Rev (Table S5). The amplicons, which contain overlapping extensions, were purified, combined and used as a template in a second-round PCR using the primers Rv3012c-EcoRI-For/ guaB3-EcoRI-Rev. The full-length PCR product was purified, restricted with *Eco*RI, and cloned in pTT1B-Gm to produce pTTguaB3, which was integrated into

guaB2-guaB3 Tet-OFF to produce the complemented mutant, *guaB2-guaB3* Tet-OFF *attB*::*guaB3*.

Western blot analysis

Cultures of *Mtb* strains were grown to an OD_{600} of 0.5 in Middlebrook 7H9 broth supplemented with antibiotics, as required, treated with ATc (10 ng/mL) and incubated at 37°C for 7 d. Cultures (5 mL) were harvested by centrifugation at selected time points (1, 2, 3, 5 and 7 d), washed twice with PBS (pH 7.5), and resuspended in 1 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 1× protease inhibitors and 10% glycerol). The re-suspended cells were transferred to a Lysing Matrix B tube (MP Biomedicals) and processed in a FP120 bead beater (MP Biomedicals) for two rounds of 40 s pulses at a speed of 6.5 with an incubation interval of 5 min on ice between each pulse. The processed samples were centrifuged and the supernatants were filtered twice using Costar Spin-X Centrifuge 0.22 µm Tube Filter (Corning, USA). The protein concentration of each lysate was quantified by a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific), using supplied reagents and standards. Twenty-five µg of each lysate was fractionated by SDS-PAGE and analysed by Western blot using ECL Select Western Blotting Detection Reagent (Amersham). The primary GuaB2 antibody (mouse, Alere™)[10] and the secondary goat anti-mouse Ig, kappa LC Ab peroxidase conjugate (Pierce) were used at dilutions of 1:5000 and 1:100000, respectively.

Gene expression analysis by ddPCR

Total RNA from *Mtb* cultures was extracted using a FastRNA ProBlue Kit (MP Biomedicals), as per the manufacturer's instructions, followed by two rounds of DNase treatment of RNA using Turbo DNase (Ambion). DNase-treated RNA (100 ng) was used as template for cDNA synthesis using iScript™ cDNA synthesis kit (Bio-Rad). Primers and

TaqMan minor groove binder (MGB) probes (Table S5) were designed using Primer Express software (version 3.0.1). To facilitate the multiplexing of the assay, TaqMan MGB probes homologous to the target gene were labelled with 6-carboxyfluorescein (FAM) whereas the reference gene, *sigA*, was labelled with 4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein (VIC). The PCR was performed as described previously[1] cycling at 95°C for 10 min; 40 cycles of 94°C for 30 s and 60°C for 60 s; and 98°C for 10 min in a conventional T100 Thermal Cycler (Bio-Rad), after which the droplets were analysed using a QX200 Droplet Reader (Bio-Rad). Three technical replicates of each gene were run for each biological replicate. The analysis was performed as described previously[1].

Analysis of cidality caused by GuaB2 depletion *in vitro*

To test the *guaB2* silencing effect on viability of bacteria, the *guaB2-guaB3* Tet-OFF *attB::guaB3* and H37Rv strains were grown at 37°C in 7H9 broth supplemented with OADC, glycerol and Tween 80 to mid-exponential phase and then standard inoculum (~5×10^4 CFU/mL) was added to 7H9 broth (with OADC and Tween80) containing presence (10 ng/mL) or absence of ATc. The viability was assessed by plating serial dilutions at days 0, 1, 2, 5, 7, 10 and 12 on standard 7H10 agar containing the required antibiotics (50 µg/mL Hyg, 25 µg/mL Km and 2.5 µg/mL Gm; for *guaB2-guaB3* Tet-OFF *attB*::*guaB3*), with or without ATc (200 ng/mL), and with or without guanine (200 µM). Aliquots (100 µL) withdrawn for CFU enumeration were washed with 1 mL of 7H9 broth to remove residual ATc and resuspended in 1 mL of fresh 7H9 broth prior to plating. Plates were incubated for 3-4 weeks before scoring CFUs.

Macrophage infections

THP-1 cells were obtained from ATCC collection, and handled and infected, as previously described.[11] Briefly, the cells were grown in standard RPMI-1640 medium (Sigma-Aldrich) containing 10% foetal bovine serum (HyClone), and incubated at 37°C in 5% CO_2 environment. After expansion, the THP-1 cells were pelleted by centrifuging at 200 × *g* for 10 min at room temperature, re-suspended in warm RPMI and the cell density adjusted to 7.5 × 10^5 cells/mL. Phorbol 12-myristate 13-acetate (50 nM, Sigma-Aldrich) was added to the cells suspension before dispensing 100 µL of ~7.5 x 10^5 cells per mL into wells of 96-well tissue culture plate and incubating for 24 h, to allow differentiation into macrophages. After removal of the media, the cells were washed once with warm PBS (Sigma-Aldrich) and treated with 100 µl RPMI containing ~3.7 x 10^3 CFU/mL of H37Rv or *guaB2-guaB3* Tet-OFF *attB*::*guaB3* (MOI = 1:20 CFU per macrophage) for 4 hours at 37°C, to allow the macrophages to phagocytose the bacteria. Under these experimental conditions, ~ 50% of the bacteria were internalized by macrophages. The infected macrophages were washed twice with warm PBS to remove extracellular bacteria and grown in standard RPMI media with or without ATc (200 ng/mL) with various concentrations of guanine (10, 50, 100, or 200 µM), and incubated at 37°C for 7 days, changing media every 48 h. At various time points, the macrophages were lysed with 100 µL of 0.05% SDS to release the bacteria. This treatment had no effect on the viability of bacteria, as determined by CFU enumeration. The re-suspension was immediately diluted in 1 mL of fresh 7H9, centrifuged, and the supernatant discarded to remove any residual ATc or guanine, before re-suspension in 1 mL fresh 7H9, and plating of serial dilutions on Middlebrook 7H10-OADC containing required antibiotics (50 µg/mL Hyg, 25 µg/mL Km and 2.5 µg/mL Gm; for *guaB2-guaB3* Tet-OFF *attB*::*guaB3*), with or without ATc (200 ng/mL) or guanine (200 µM). Viability of the macrophages infected with H37Rv or the conditional mutant was measured by exposing the infected cells to PrestoBlue Cell Viability Reagent (Life Technology) for 1 h. Fluorescence was read using

FLUOstar OPTIMA plate reader (BMG LABTECH, Offenberg, Germany). Approximately 95% of macrophages remained viable during the experimental time course (7 days).

Cloning, expression and purification of wildtype and Y487C *Mtb* GuaB2 and *Mth* GuaB2

The *Rv3411c* gene encoding *Mtb* GuaB2 was PCR amplified (Table S5) from *Mtb* H37Rv genomic DNA, and cloned into a pCold™ I DNA vector (Takara) by conventional methods with an N-terminal His6tag. Recombinant *Mtb* GuaB2 was expressed in auto-inducing ZYP-5052 medium in the presence of ampicillin (50 µg/mL), and chloramphenicol (34 µg/mL). The constructed plasmid was transformed into *E. coli* BL21 (DE3). A single colony was inoculated into 20 mL of 2×TY with the same antibiotic and incubated overnight at 37ºC. The culture was transferred into 1 L of auto-inducing ZYP-5052 medium in a 5 L shaking flask, grown at 37ºC to an OD_{600} = 2, and then grown at 17ºC for a further 20 h. Cultures were harvested by centrifugation, and cells were re-suspended in Buffer A (50 mM Tris-HCl pH 8.0, 500 mM KCl, 2.5 mM DTT, 10% Glycerol) supplemented with protease inhibitor cocktail, and benzonase nuclease (Sigma-Aldrich), and then lysed by sonication on ice. The lysate was clarified by centrifugation, and the soluble fraction was loaded onto a Ni-NTA column equilibrated with Buffer A. After an extensive washing step with Buffer A, a second washing step is done with ten column volumes of Buffer A supplemented with 80mM imidazole, then the bound His-tag N-terminal enzyme was eluted with Buffer A supplemented with 500 mM imidazole, and fractions containing *Mtb* GuaB2 were pooled and loaded on a HiPrep 26/10 desalting column with Buffer A. The resulting protein was collected and concentrated to 0.5 mg/mL. All the purification steps above described were performed at 4ºC. Concentrations were determined by Bradford assay, and sample purity was assessed by 10% SDS-PAGE.

The construct coding for wildtype *Mtb* GuaB2, obtained as detailed above, was used as the DNA template in a PCR-based site-directed mutagenesis experiments, using the QuikChange II site directed mutagenesis kit reagents (Stratagene) and the primer pairs Y487C_forward 5'-gcgacccggtg**c**agcccatcgcg-3' and Y487C_reverse 5'-cgcgatgggct**g**caccgggtcgc-3'. The region encoding the mutant *Mtb* GuaB2 variant in the resulting expression construct was verified by sequencing (Eurofins MWG Operon). The expression and purification of *Mtb* GuaB2 carrying the Y487C mutation was carried out using the same procedure as for the wildtype enzyme, as described above.

The gene encoding *Mth* GuaB2 was amplified from genomic DNA. In order to facilitate protein expression and crystallisation, the GuaB2 gene was cloned into the pHat2 vector after removing the 2 CBS domains and replacing with a GG linker connecting the two parts of the catalytic region (*Mth* GuaB2 ΔCBS). The pHat2 vector has an N-terminal His_6-tag and a TEV site was included in the construct to enable affinity-tag removal. The *Mth* GuaB2 ΔCBS protein was expressed in BL21 DE3 (NEB) cells at 37°C until the OD_{600} reached 0.6, then expression was induced by reduction of the temperature to 18°C and addition of IPTG at a final concentration of 500 μM. Cells were left growing overnight; after which they were harvested by centrifugation, resuspended in 50 mM Hepes pH 8.0, 500 mM NaCl, 5% glycerol, 10 mM β-mercaptoethanol, 20 mM imidazole and lysed by 3 passes through an Emulsiflex cell disruptor (Avastin). The resulting lysate was clarified by high-speed centrifugation and filtration through a 0.45 μm filter. This was then applied to a Hi-Trap IMAC FF column (GE Healthcare) charged with Nickel. The bound protein was eluted with lysis buffer + 250 mM imidazole. The elution fractions were pooled and dialysed overnight into lysis buffer – imidazole. In order to remove the N-terminal His-tag the protein was incubated with TEV protease during dialysis. A negative nickel gravity-flow purification step was performed to remove the uncleaved protein and the protease. The flow-through from

this step was concentrated and applied to a Superdex 200 gel filtration column pre-equilibrated with 20 mM Hepes pH 8.0, 500 mM NaCl, 5% glycerol, 1 mM TCEP. Elution fractions were collected and concentrated to 12.5 mg/mL for crystallisation.

Enzyme kinetics

Recombinant *Mtb* GuaB2 was prepared as described above and human IMPDH was purchased from Sigma-Aldrich. The enzymatic activity was measured by a continuous spectrophotometric assay[12] in a 200 µl reaction mixture that contained 50 mM Tris.HCl buffer (pH 8.0), 150 mM KCl, 1 mM DTT, 1 mM EDTA, 3 mM NAD^+ and 1.25 mM IMP (all chemicals were purchased from Sigma-Aldrich). After 1 minute of pre-incubation, the reaction was started by adding 0.5 µg of either the *Mtb* or human enzyme and the increase in the absorbance at 340 nm, caused by the reduction of NAD^+ to NADH (ε_{340} = 6220 M^{-1} cm^{-1}), measured. The assay was performed in quartz cuvettes with a Varian Cary 50-BiO UV-visible spectrophotometer equipped with a temperature controlled cuvette holder.

The apparent steady-state parameters for *Mtb* GuaB2 on its natural substrates were collected using the continuous spectrophotometric assay described previously [12]. IMP and NAD^+ concentrations when held constant were at 1.25 mM and 3 mM, respectively. The concentrations of IMP were varied from 0.01 mM to 0.6 mM, and for NAD^+, were varied from 0.1 mM to 3 mM. Data points were obtained from three independent experiments. Kinetic parameters were calculated by using a nonlinear least square fit of the data using Sigma Plot-Enzyme Kinetics Module 1.3. IMP, and NAD^+ data were fitted with Equation 1 (the Michaelis-Menten equation for hyperbolic substrate kinetics).

$$v=V_{max} \times S/(K_M+S) \quad \text{(Eq. 1)}$$

In which v, V_{max}, S, and K_M represent, respectively, steady state reaction rate (μmol min^{-1} mg^{-1}), maximum reaction rate, substrate concentration, and Michaelis-Menten constant for S.

For the enzyme inhibition study, the concentration of VCC234718 required to reduce the fractional enzyme activity to half of its initial value in the absence of inhibitor (IC_{50}) was calculated plotting the *Mtb* GuaB2 or human IMPDH fractional activity against the logarithm of inhibitor concentration, and fitting the curves to a dose response curve (Equation 2):

$$y=min+ ((max-min)/(1+10^{(LogIC50-x)})) \quad (Eq.2)$$

in which y is the fractional activity of the enzyme in the presence of inhibitor at concentration [I], max is the maximum value of y observed at [I]=0, and min is the minimum limiting value of y at high inhibitor concentrations. The measured K_i against *Mtb* GuaB2 was calculated by analysis of the initial velocity data plotted against the substrate concentration. The data were fitted to equation describing the uncompetitive inhibition model using Sigma Plot-Enzyme Kinetics Module 1.3. The concentrations of VCC234718 were varied from 0 to 0.2 μM. Data points were obtained from three independent experiments. In the case of *Mtb* $GuaB2^{Y487C}$, no effect on the enzymatic activity could be detected up to a VCC234718 concentration of 100 □M.

Table S1. Pharmacological properties of VCC234718

Assay	Value
Permeability Caco2 assay [P Total (10^{-7} cm.s^{-1}), % recovery]	297.7±1.4, 87%
CYP3A4 inhibition in human liver microsomes [IC_{50} (µM)] substrate = testosterone	<1.0
CYP3A4 inhibition in human liver microsomes [IC_{50} (µM)] substrate = midazolam	1.30
Intrinsic clearance and CYP3A contribution in human primary hepatocytes, at 5µM [CL_{int} (mL/h/10^6 cells), % CYP3A contribution]	2.2, 0%
Intrinsic clearance in rat hepatic microsomal fractions [clearance (µL/min/mg protein)]	>1000
Intrinsic clearance in mouse hepatic microsomal fractions [clearance (µL/min/mg protein)]	>1000
Intrinsic clearance in human hepatic microsomal fractions [clearance (µL/min/mg protein)]	642
Human ERG antagonist activity [IC50 (µM)]	13.7±1.1

Table S2. Mutations identified in VCC234718-resistant mutants of *Mtb*

Genome Position	Resistant mutant	Resistance level	Gene	Base change	Amino acid change
779239	SRMV2.10	4×	*rv0678*[#]	g250a	A84T
3245354	SRMV2.3 & 2.10	4-8×	*fadD26*[#]	c1658a	S553*
3248028	SRMV2.6	100×	*ppsA*[#]	g2584a	V862M
3830060	SRMV2.6	100×	*guaB2*	a1460g	Y487C
3898312	SRMV2.3	8×	*rv3479*[#]	c2493a	N831K
4201590	SRMV2.10	4×	*rv3755c*[#]	a299t	D100G

[#] Non-essential gene [13]

Table S3. Steady-state kinetic parameters for *Mtb* GuaB2 and inhibition constant (*K*i) for VCC234718, determined as described under Methods.

Substrate	**K_m (μM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_m ($s^{-1}/\mu M^{-1}$)**	**K_i (μM)**
IMP	85 ± 16	0.80 ± 0.06	9.40 ± 0.90 ($\times 10^{-3}$)	0.12 ± 0.02
NAD^+	940 ± 31	0.90 ± 0.03	9.60 ± 0.17 ($\times 10^{-4}$)	0.10 ± 0.01

Table S4. Data collection and refinement statistics (PDB ID: 5J5R)

Wavelength (Å)	0.9686 Å
Resolution range (Å)	44.7 - 1.6 (1.64 - 1.6)
Space group	I 4
Unit cell	89.4 89.4 84.9 90 90 90
Total reflections	290035 (21363)
Unique reflections	43941 (3238)
Multiplicity	6.6 (6.6)
Completeness (%)	100.0 (100.0)
I/sigma(I)	12.8 (1.7)
Wilson B-factor	18.4
R-merge	0.077 (1.11)
R-meas	0.092 (1.33)
CC1/2	0.998 (0.476)
R-work	0.17 (0.29)
R-free	0.19 (0.30)
Number of non-hydrogen atoms	2677
Macromolecules	2346
Ligands	50
Water	281
Protein residues	333
RMS(bonds)	0.007
RMS(angles)	1.08
Ramachandran favored (%)	99
Ramachandran outliers (%)	0
Average B-factor	27.10
Macromolecules	25.90
Ligands	27.00
Solvent	37.30

Statistics for the highest-resolution shell are shown in parentheses.

Table S5. Bacterial strains and plasmids used in this study

Name	Description	Source/ reference
Strains		
E. coli		
DH5α	F- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(rk-, mk+) *phoAsupE44 thi-1 gyrA96 relA1* λ^- ; used for transformation	Invitrogen
Mtb		
H37RvMA	Mtb H37Rv isolate from the laboratory of Dr. C. Sassetti; ATCC 27294 virulent laboratory strain	[14]
SRMV2.6	VCC234718^R mutant of H37RvMA carrying a nsSNP a1460g which changed the terminal amino acid of IMPDH domain (Tyr487Cys, Y487C) of GuaB2 (Rv3411c)	This study
SRMV2.6 *attB::guaB2*	Derivative of SRMV2.6 carrying pTTguaB2; Gm^R	This study
SRMV2.6 *attB::guaB2-guaB3*	Derivative of SRMV2.6 carrying pTTguaB2-guaB3; Gm^R	This study
H37Rv *attB::guaB2*	Derivative of H37Rv carrying pTTguaB2; Gm^R	This study
H37Rv *attB::guaB2-guaB3*	Derivative of H37Rv carrying pTTguaB2-guaB3; Gm^R	This study
SRMV2.6 *attB:: guaB2*^{Y487C}	Derivative of SRMV2.6 carrying pTTGuaB2^{Y487C}; Gm^R	This study
H37Rv *attB::guaB2*^{Y487C}	Derivative of H37Rv carrying pTTGuaB2^{Y487C}; Gm^R	This study
guaB2-guaB3-SCO	Derivative of H37Rv in which expression of *guaB2-guaB3* is controlled by P_{myc1}tetO ; Hyg^R	This study
guaB2-guaB3 Tet-ON	Derivative of *guaB2-guaB3*-SCO carrying pGMCK-OX21-T10 integrated at the L5 *attB* site; Hyg^R, Km^R	This study
guaB2-guaB3 Tet-OFF	Derivative of *guaB2-guaB3*-SCO carrying pGMCK3-OX38-T28 integrated at the L5 *attB* site; Hyg^R, Km^R	This study
guaB2-guaB3 Tet-OFF *attB::guaB3*	Derivative of *guaB2-guaB3* Tet-OFF carrying pTTguaB3; Hyg^R, Km^R, Gm^R	This study
Plasmids		
pTT1B::Gm	Gentamicin-resistant derivative of the Tweety-based integration vector, pTTP1B; Gm^R, Amp^R	[2]
pTTguaB2	*Mtb guaB2* complementation vector– pTT1B::Gm carrying 1920 bp region of the H37Rv chromosome which includes the full-length *guaB2* gene and 330 bp of upstream sequence; Gm^R	This study
pTTguaB2-guaB3	Mtb *guaB2-guaB3* complementation vector– pTT1B::Gm carrying 3086 bp region of the H37Rv chromosome which includes the full-length *guaB2-guaB3* gene and 330 bp of upstream sequence; Gm^R	
pTTguaB2^{Y487C}	*Mtb guaB2*^{Y487C} complementation vector– pTT1B::Gm carrying 1920 bp region of the SRMV2.6 chromosome which includes the full-length *guaB2*^{Y487C} gene and 330 bp of upstream sequence; Gm^R	This study

pSE100	*E. coli-Mycobacterium* shuttle vector carrying P_{myc1}*tetO*; Hyg^R	[7]
pguaB2-guaB3-SCO	Suicide plasmid for generating *guaB2-guaB3*-SCO. pSE100 derivative in which the mycobacterial origin of replication was replaced by 722 bp of *guaB2* region which includes 15 bp of upstream and the first 707 bp of *guaB2;* Hyg^R	This study
pGMCK-OX21-T10	L5-based integration vector harbouring P_{imyc}-tetR; Km^R	[8]
pGMCK3-OX38-T28	L5-based integration vector harbouring modified P_{smyc}-tetR r1.7; Km^R	[8]
pGA0XP15-intL5	Suicide vector harbouring L5 integrase; Amp^R	[8]
pTTguaB3	Mtb *guaB3* complementation vector– pTT1B::Gm carrying 1547 bp region of the H37Rv chromosome which includes the 382 bp of upstream sequence of *guaB2* gene, 19 bp of intergenic region between *guaB2* and *guaB3*, and full-length *guaB3* gene with additional 18 bp of downstream; Gm^R	This study

Table S6. PCR primers, other oligonucleotides and amplicons used in this study

Name	Sequence (5'-3')[a]	Application
guaB2 seq F1	ttgagttctcgcttcgtgt	Primer used for the sequencing of *guaB2*
guaB2 seq F2	gtcgagatggtgaagcgat	Primer used for the sequencing of *guaB2*
guaB2 seq R1	ttcacgaagatcagctcac	Primer used for the sequencing of *guaB2*
guaB2 seq R2	caccactgggatctcgaac	Primer used for the sequencing of *guaB2*
guaB2 CF	tacccg**gaattc**cggccatccgctcttgttgat	Forward primer used for the complementation of *guaB2* or *guaB2*^{Y487C} or *guaB2-guaB3*
guaB2 CR	tacccg**gaattc**tcattctagcttacgttagcgcgcgtagtagttgg	Reverse primer used for the complementation of *guaB2* or *guaB2*^{Y487C}
guaB2-B3 CR	tacccg**gaattc**tcattctagcttacgatcagtctctcaggtggcgt	Reverse primer used for the complementation of *guaB2-guaB3*
guaB2-B3_*Sph*I F	cgctgg**gcatgc**tggagggccccaccgatg	Forward primer used to amplify a 5'-fragment of the *guaB2* gene
guaB2-B3_*Not*I R	gtccaa**gcggccgc**tcaaagtccttgacggtgatc	Reverse primer used to amplify a 5'-fragment of the *guaB2* gene
guaB2 SCO F	ggatgggcttctcactga	To confirm the SCO genotype
guaB2 SCO R	gctgagttcataggtgcg	To confirm the SCO genotype
guaB2 probe For	ctggaagacagctccgac	Forward primer for amplifying probe (Southern)
guaB2 probe Rev	ctcggcgacctgcttgga	Reverse primer for amplifying probe (Southern)
Rv3012c-EcoRI-For	tacccg**gaattc**ttgtgcgccaacagagct	Primers for *guaB3* complementation
guaB2'-SOE-Rev	cggtggggccctccatacgcat	Primers for *guaB3* complementation
guaB3-SOE-For	atgcgtatggagggccccaccgcggcgacctggatatctaga	Primers for *guaB3* complementation
guaB3-EcoRI-Rev	tacccg**gaattc**tcagtctctcaggtggcgtc	Primers for *guaB3* complementation
guaB2RTF	aacggcaagcagtacaagagttatc	Forward primer for ddPCR analysis of *guaB2*
guaB2RTR	Gaagtagcgatccttggaatacga	Reverse primer for ddPCR analysis of *guaB2*
guaB2probe (FAM)	Ccatgcgagggcg	TaqMan (FAM) probe for ddPCR of *guaB2*
guaB3RTF	Ggtgagcccgcaaaacg	Forward primer for ddPCR analysis of *guaB3*

guaB3RTR	tggatgaccagcaagtcaatacc	Reverse primer for ddPCR analysis of *guaB3*
guaB3Probe (FAM)	tgacaccggtactggtt	TaqMan (FAM) probe for ddPCR of *guaB3*
sigARTF	cgagccgatctcgttgga	Forward primer for ddPCR analysis of *sigA*
sigARTR	ttcgatgaaatcgccaagct	Reverse primer for ddPCR analysis of *sigA*
sigAProbe (VIC)	acgagggcgacagc	TaqMan (VIC) probe for ddPCR of *sigA*
pSE100 F	tgagctctacgccatcccg	Primers to verify the cloned gene in pSE100 and to confirm homologous recombination of pguaB2-guaB3-SCO
pSE100 R	tctccggcttcaccgatcc	
rv3411c-Fw	gatcgatc**catatg**tcccgtggcatgtccggc	Forward primer for expression of recombinant *Mtb* GuaB2
rv3411c-Re	gatcgatc**aagctt**ttagcgcgcgtagtagttggg	Reverse primer for expression of recombinant *Mtb* GuaB2

[a]Restriction sites are shown in bold; in-frame stop codons are underlined.

FIGURES

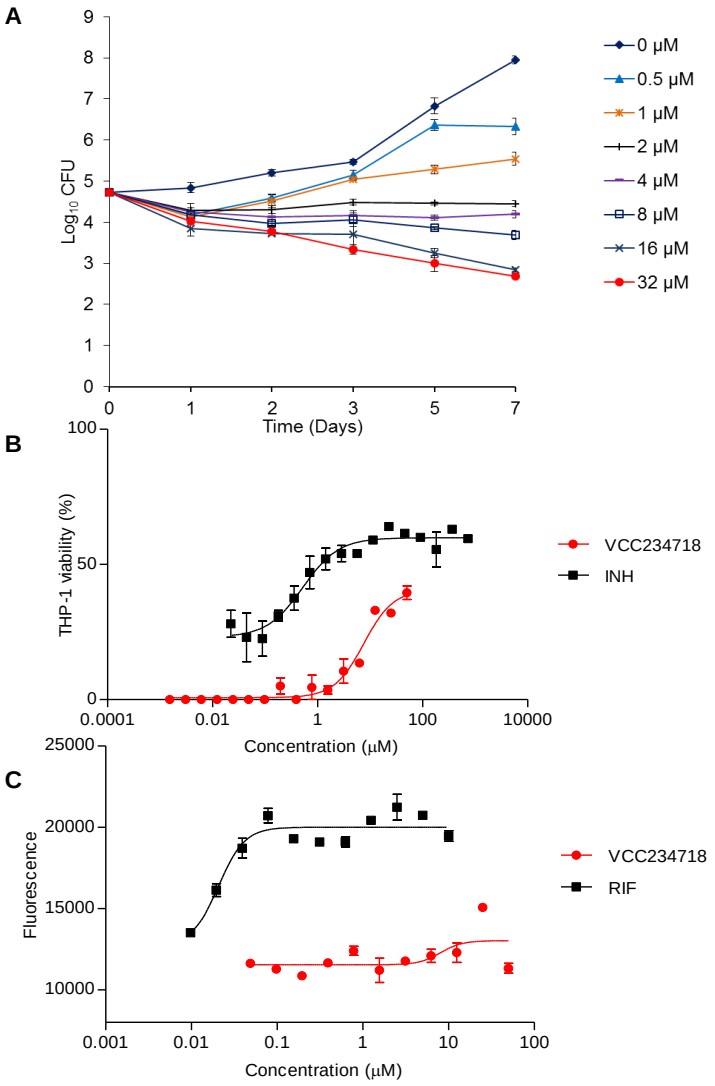


Figure S1. The effect of VCC234718 on viability of *Mtb in vitro* (A), TB-infected THP-1 macrophages (B) and MRC-5 human lung fibroblasts (C). (A) The time-kill kinetic analysis was performed by exposing cultures to varying concentrations of compound and scoring CFU at the indicated time points. The results are shown as mean values and standard deviation from three replicates. (B) THP-1 cells were differentiated by treatment overnight with PMA. Cells were infected with *Mtb* H37Rv at a MOI of 5 and incubated for 48 h in the presence of VCC234718 or isoniazid (INH). Results were normalized to untreated H37Rv-infected THP-1 cells (approx. 20% killing). (C) MRC-5 fibroblasts were infected with *Mtb* Erdman at a MOI of 10 and exposed to VCC234718 or rifampicin (RIF) for 72 h. Data are expressed as the mean values and standard deviation of two individual experiments. Viable fibroblasts and macrophages were quantified using Prestoblue.

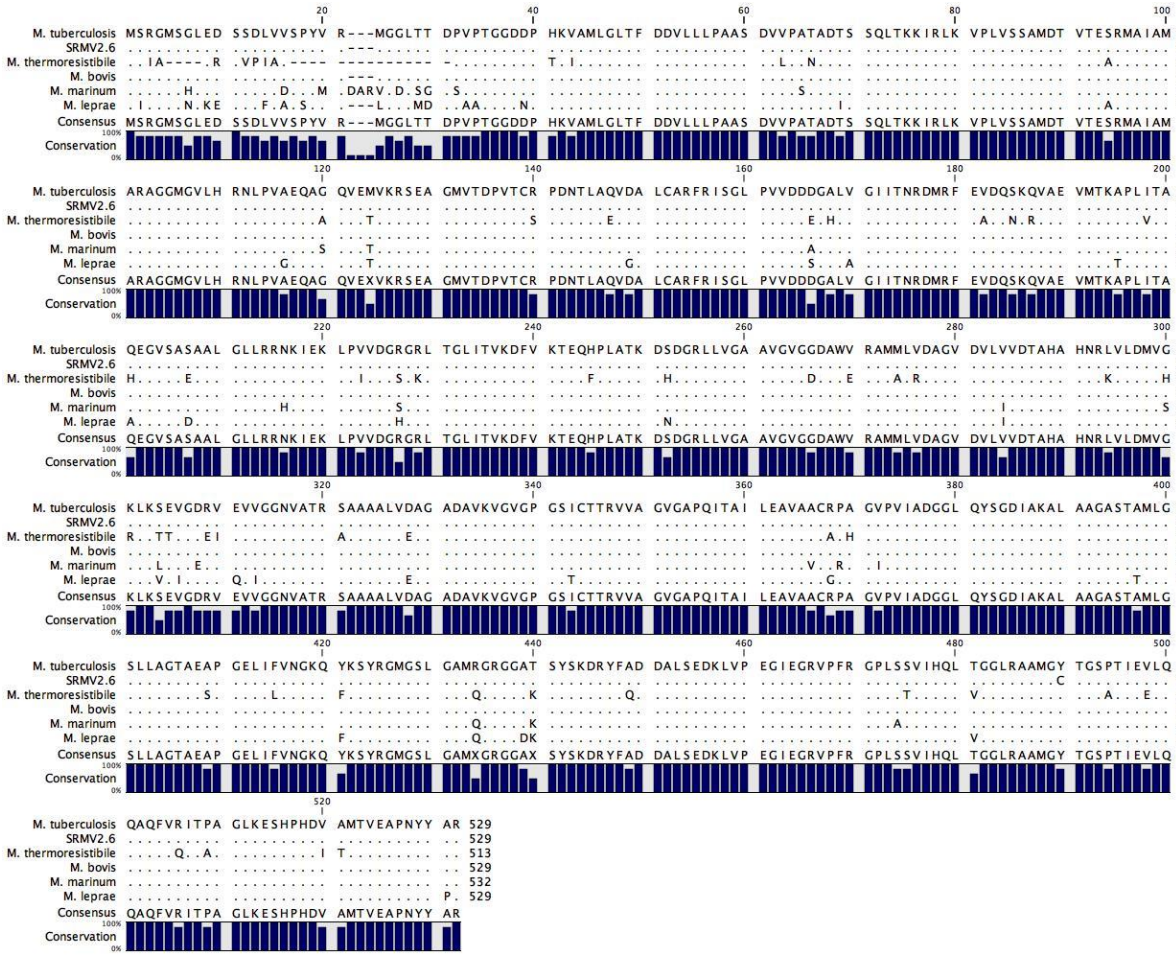


Figure S2. Sequence comparison of GuaB2 from wildtype *Mtb*, SRMV2.6, *M. thermoresistibile*, *M. bovis*, *M. marinum*, and *M. leprae*. The amino acid sequence alignment was generated using CLC DNA Workbench version 6.0.2 (CLC bio). The amino acid residue circled in red is mutated in the SRMV2.6 (VCC234718-resistant) mutant of *Mtb*. Conserved amino acids are represented as a dot. Conservation levels are displayed as a bar graph at the bottom of the alignment. The bar shows the conservation of all sequence positions where the height of the graph (on a scale of 0-100 %) reflects how conserved that particular position is in the alignment.

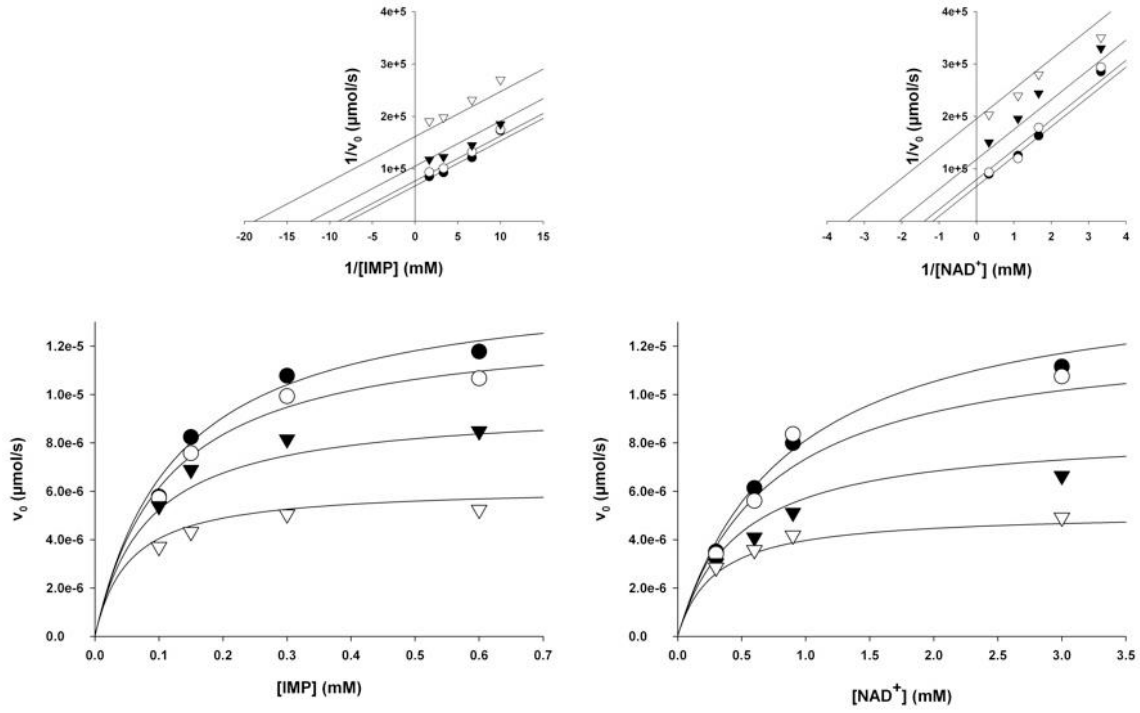


Figure S3. The mechanism of inhibition of VCC234718. Details the experimental conditions and the way in which the data were fitted, are described under "Enzyme kinetics" in the Methods section above. The Lineweaver-Burke plots (upper) were generated to display the type of inhibition, which is uncompetitive for both IMP and NAD^+.

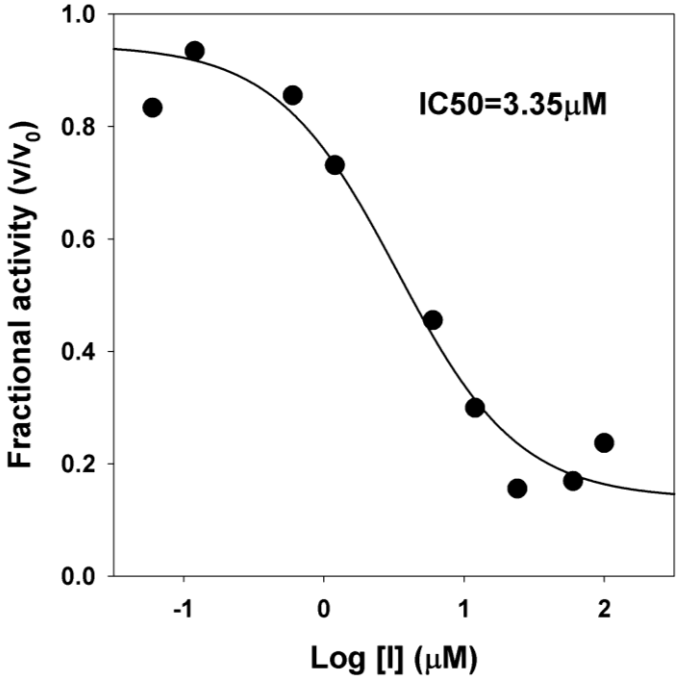


Figure S4. Inhibition of human IMPDH by VCC234718. The graph depicting the enzyme fractional activity *versus* the logarithm of inhibitor concentration was used to calculate IC_{50} of VCC234718 against human IMPDH.

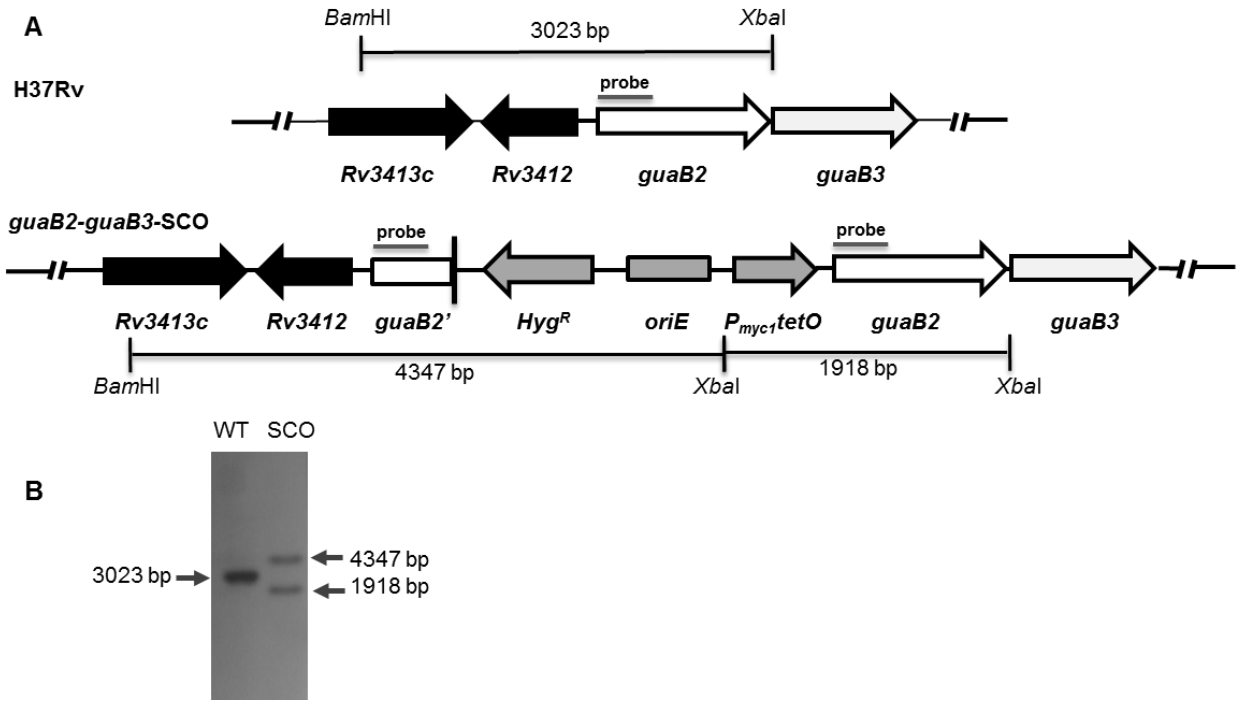


Figure S5. Genotypic characterisation of *guaB2-guaB3-SCO*. (A) Map of the chromosomal locus of *guaB2* in wild type *Mtb* and the *guaB2-guaB3*-SCO promoter-replacement mutant. (B) Southern blot analysis of *Bam*HI and *Xba*I digested genomic DNA probed with a fragment corresponding to the 5'-terminal 486 bp of the *guaB2* gene.

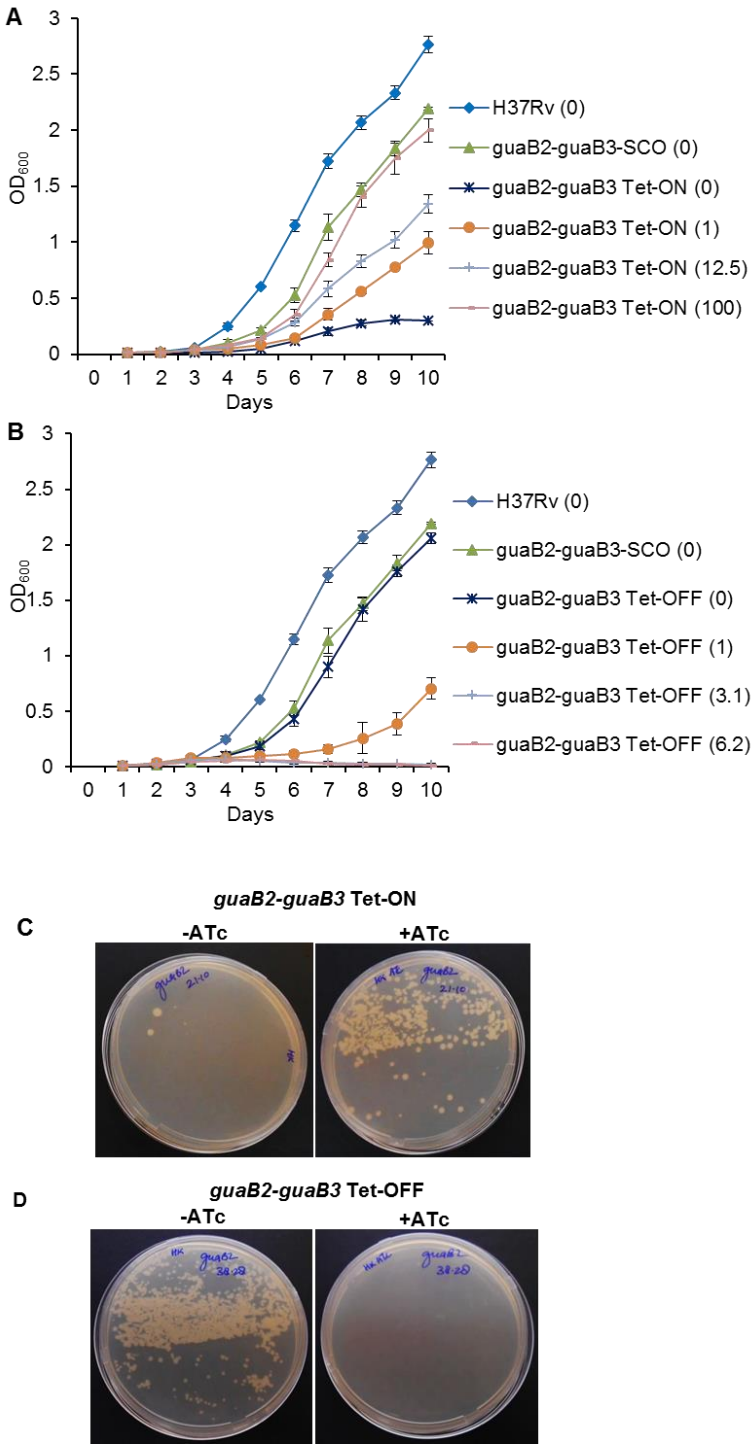


Figure S6. ATc-dependent growth of conditional *guaB2-guaB3* mutants. Growth of *guaB2-B3* Tet-ON_M (A) and *guaB2-B3* Tet-OFF (B) was monitored in Middlebrook 7H9 medium supplemented with the indicated concentrations of ATc using the and *guaB2-guaB3*-SCO strains as controls. Data are means ± SD of three independent experiments. Growth of the *guaB2-guaB3* Tet-ON mutant is suppressed in the absence of ATc in liquid medium (A) or on agar (C), whereas growth of the *guaB2-guaB3* Tet-OFF mutant is suppressed in the presence of ATc in liquid medium (B) or on agar (D).

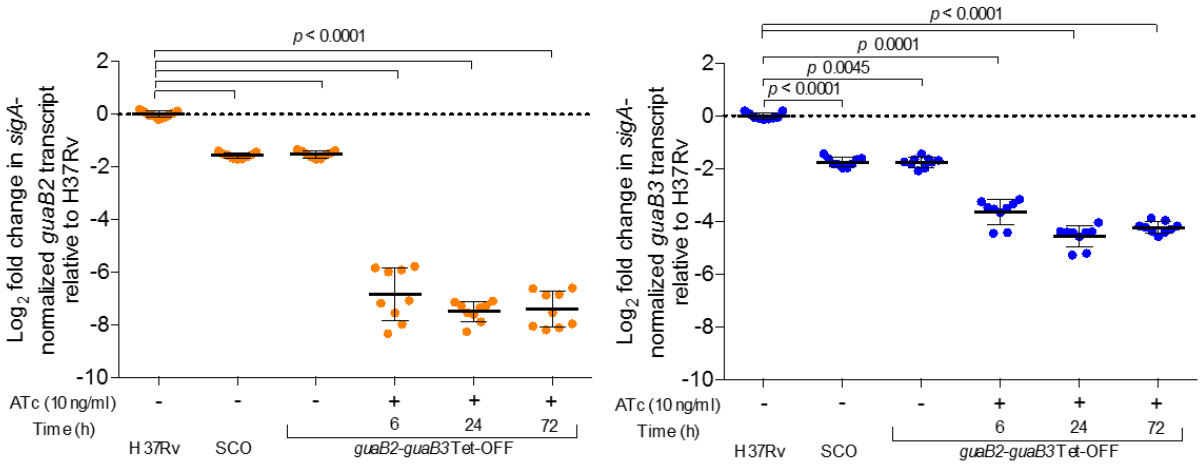


Figure S7. Transcript analysis of *guaB2* and *guaB3* relative to the *sigA* reference in exponentially grown (OD_{600} = 0.5) *guaB2-guaB3* Tet-OFF strain in response to ATc.

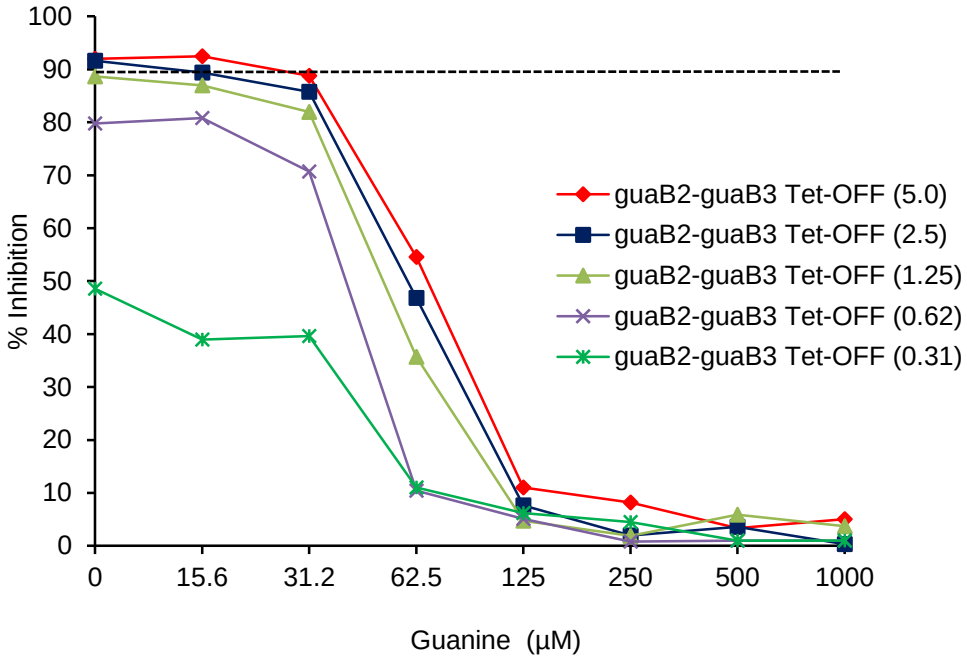


Figure S8. Guanine rescue of ATc-mediated growth inhibition of *guaB2-guaB3* Tet-OFF.

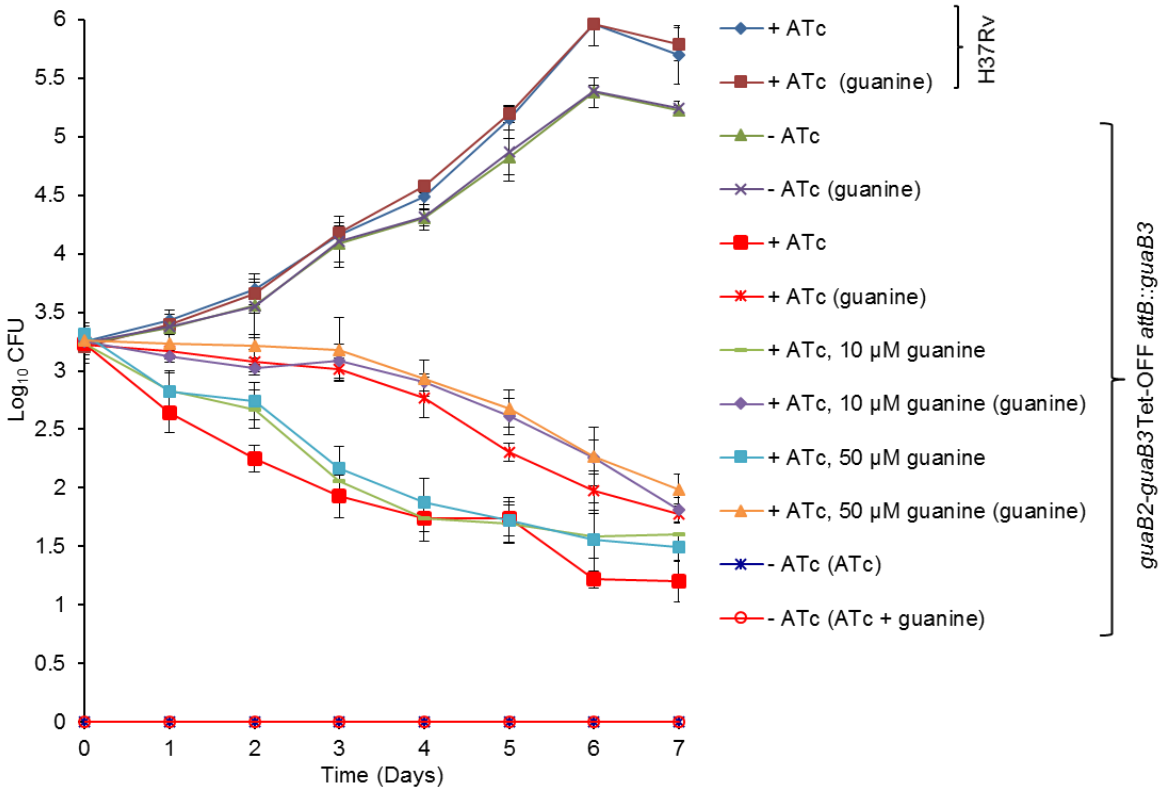


Figure S9 (related to Figure 6A). Depletion of GuaB2 is bactericidal *ex vivo*. The experiment was performed as described in the Methods and Supplementary Information. The results represent the mean ± SD from three biological replicates. The plating conditions are shown in parentheses in the legend.

Movie S1 & S2. Time-lapse microscopy of *M. tuberculosis* and the effect of transcriptional silencing of *guaB2*. The *guaB2-guaB3* Tet-OFF *attB::guaB3* strain was cultured in a microfluidic device as described in the Methods section and imaged at 15 min intervals. Medium was supplemented with 100 ng/ml ATc between 74-379 h and with 125 µM guanine between 334-500 h. Images were recorded on phase. Numbers (top left) indicate time elapsed. Labels (top right) indicate the medium supplements. Scale bar (5 µm) is indicated on bottom left. Snapshots at some time points which were out-of-focus were removed when assembling the movie. Movie S1 is a representative movie where none of the surviving cells recover growth on ATc withdrawal, whereas Movie S2 is a representative example, where some of the survivors were able to grow and divide on guanine supplementation.

REFERENCES

1. Singh, V.; Brecik, M.; Mukherjee, R.; Evans, J. C.; Svetlikova, Z.; Blasko, J.; Surade, S.; Blackburn, J.; Warner, D. F.; Mikusova, K.; Mizrahi, V., The complex mechanism of antimycobacterial action of 5-fluorouracil. *Chem Biol* **2015,** *22* (1), 63-75.

2. Abrahams, G. L.; Kumar, A.; Savvi, S.; Hung, A. W.; Wen, S.; Abell, C.; Barry, C. E., 3rd; Sherman, D. R.; Boshoff, H. I.; Mizrahi, V., Pathway-selective sensitization of *Mycobacterium tuberculosis* for target-based whole-cell screening. *Chem Biol* **2012,** *19* (7), 844-54.

3. Sala, C.; Dhar, N.; Hartkoorn, R. C.; Zhang, M.; Ha, Y. H.; Schneider, P.; Cole, S. T., Simple model for testing drugs against nonreplicating *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **2010,** *54* (10), 4150-8.

4. Vocat, A.; Hartkoorn, R. C.; Lechartier, B.; Zhang, M.; Dhar, N.; Cole, S. T.; Sala, C., Bioluminescence for assessing drug potency against nonreplicating *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **2015,** *59* (7), 4012-9.

5. Rybniker, J.; Chen, J. M.; Sala, C.; Hartkoorn, R. C.; Vocat, A.; Benjak, A.; Boy-Rottger, S.; Zhang, M.; Szekely, R.; Greff, Z.; Orfi, L.; Szabadkai, I.; Pato, J.; Keri, G.; Cole, S. T., Anticytolytic screen identifies inhibitors of mycobacterial virulence protein secretion. *Cell Host Microbe* **2014,** *16* (4), 538-48.

6. Ehrt, S.; Guo, X. V.; Hickey, C. M.; Ryou, M.; Monteleone, M.; Riley, L. W.; Schnappinger, D., Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. *Nucleic Acids Res* **2005,** *33* (2), e21.

7. Guo, X. V.; Monteleone, M.; Klotzsche, M.; Kamionka, A.; Hillen, W.; Braunstein, M.; Ehrt, S.; Schnappinger, D., Silencing *Mycobacterium smegmatis* by using tetracycline repressors. *J Bacteriol* **2007,** *189* (13), 4614-23.

8. Klotzsche, M.; Ehrt, S.; Schnappinger, D., Improved tetracycline repressors for gene silencing in mycobacteria. *Nucleic Acids Res* **2009,** *37* (6), 1778-88.

9. Ho, S. N.; Hunt, H. D.; Horton, R. M.; Pullen, J. K.; Pease, L. R., Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **1989,** *77* (1), 51-9.

10. Buechler, J.; Valkirs, G.; Gray, J. Polyvalent display libraries. US 6057098 A, 2000.

11. Manganelli, R.; Voskuil, M. I.; Schoolnik, G. K.; Smith, I., The *Mycobacterium tuberculosis* ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. *Mol Microbiol* **2001,** *41* (2), 423-37.

12. Usha, V.; Gurcha, S. S.; Lovering, A. L.; Lloyd, A. J.; Papaemmanouil, A.; Reynolds, R. C.; Besra, G. S., Identification of novel diphenyl urea inhibitors of Mt-GuaB2 active against Mycobacterium tuberculosis. *Microbiology-Sgm* **2011,** *157*, 290-299.

13. Griffin, J. E.; Gawronski, J. D.; Dejesus, M. A.; Ioerger, T. R.; Akerley, B. J.; Sassetti, C. M., High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PLoS Pathog* **2011,** *7* (9), e1002251.

14. Ioerger, T. R.; Feng, Y.; Ganesula, K.; Chen, X.; Dobos, K. M.; Fortune, S.; Jacobs, W. R., Jr.; Mizrahi, V.; Parish, T.; Rubin, E.; Sassetti, C.; Sacchettini, J. C., Variation among genome sequences of H37Rv strains of Mycobacterium tuberculosis from multiple laboratories. *J Bacteriol* **2010,** *192* (14), 3645-53.

This information is available free of charge via the Internet at http://pubs.acs.org/.