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Supplementary Materials for

Inhibiting the stringent response blocks *Mycobacterium tuberculosis* entry into quiescence and reduces persistence

Noton K. Dutta, Lee G. Klinkenberg, Maria-Jesus Vazquez, Delfina Segura-Carro, Gonzalo Colmenarejo, Fernando Ramon, Beatriz Rodriguez-Miquel, Lydia Mata-Cantero, Esther Porras-De Francisco, Yu-Min Chuang, Harvey Rubin, Jae Jin Lee, Hyungjin Eoh, Joel S. Bader, Esther Perez-Herran, Alfonso Mendoza-Losana, Petros C. Karakousis*

*Corresponding author. Email: petros@jhmi.edu

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Supplementary Text

Table S1. Molecular clock assay reveals that Rel_{Mtb} deficiency results in ongoing *Mtb* replication during NS.

	WT			*Δrel*			WT	*Δrel*	
Day	NS retention[a]	7H9 retention[a]	NS/7H9 ratio[b]	NS retention[a]	7H9 retention[a]	NS/7H9 ratio[b]	NS > 7H9 pvalue[c]	NS > 7H9 pvalue[c]	WT NS/7H9 > *Δrel* NS/7H9 pvalue[d]
1	1	1	1	1	1	1	-	-	-
7	0.75 (0.41 - 1.37)	0.52 (0.38 - 0.72)	1.44 (0.92 - 2.25)	0.69 (0.37 - 1.27)	0.69 (0.64 - 0.74)	1.00 (0.58 - 1.72)	0.2	0.5	0.21
14	0.69 (0.55 - 0.86)	0.35 (0.25 - 0.50)	1.78 (1.54 - 2.07)	0.79 (0.57 - 1.09)	0.73 (0.56 - 0.96)	0.97 (0.88 - 1.07)	0.037	0.4	0.02
21	0.81 (0.65 - 1.03)	0.07 (0.05 - 0.12)	9.60 (6.36 - 14.50)	0.59 (0.41 - 0.84)	0.48 (0.29 - 0.79)	1.03 (0.82 - 1.30)	0.0022	0.31	0.011

[§] Plasmid retention measured by the percentage of cells containing the plasmid are measured by CFU counting, on plates with and without selective antibiotic, shown as fold change. Error represents the standard deviation of two or three biological replicates.

[a] The fraction of cells retaining the replication clock plasmid; mean *m* and standard deviation *s* were calculated from 2-3 trials for each genotype and condition on a $\log_{10}$-scale, and for convenience are reported as arithmetic scale mean 10^m and range (10^{m-s},10^{m+s}). This +/– one standard deviation range, presented in parentheses after the mean, corresponds to the central 68.3% confidence interval.

[b] The ratio of plasmid retention for growth in nutrient starvation (NS) relative to standard medium (7H9), also calculated on a $\log_{10}$-scale and reported as arithmetic-scale mean and one standard deviation range.

[c] P-value for one-sided t-test of greater retention of plasmid in NS vs. 7H9.

Table S2. Activity of Rel_{Mtb} inhibitor candidates in enzymatic assay and in whole-cell assay against nutrient-starved *Mtb*.

Compounds	Biochemical potency against Rel_{Mtb} (pIC_{50}, μM)	*In vitro* anti-*Mtb* activity (IC50, μM)		FD
		WT	Δ*rel*	
X9	4.8	2	16	8
H1	5.1	4	32	8
J1	4.8	8	>64	8
A1	5.3	8	>128	16
E1	5.4	8	64	8
X12	5	0.5	4	8
A4	4.2	2	16	8
X8	4.9	8	64	8
J5	5	8	>64	8
J4	4.9	4	32	8
K2	4.9	4	>64	16

All compounds shown have at least an 8-fold increase in activity against wild type (WT) vs. Δ*rel*. IC_{50} results were replicated in triplicate. FD = fold difference between IC_{50} for WT and IC_{50} for Δ*rel*.

Table S3. X9 significantly reduced the MBC of INH (in μg/ml) against *Mtb* during NS.

[GSK-X9]	wild-type	Δ*rel*	*rel* Comp
0 μM	61.44	0.96*	61.44
0.5 μM	30.72	0.96*	15.36
1 μM	3.84	0.96*	15.36
2 μM	15.36	0.96*	15.36
4 μM	3.84	0.96*	3.84

Samples were incubated, in the presence of X9, in non-shaking tubes containing 1x NS for 7 days at 37°C prior to addition of INH. CFU were plated after 7 days of INH exposure. * = lowest concentration of INH tested in these assays.

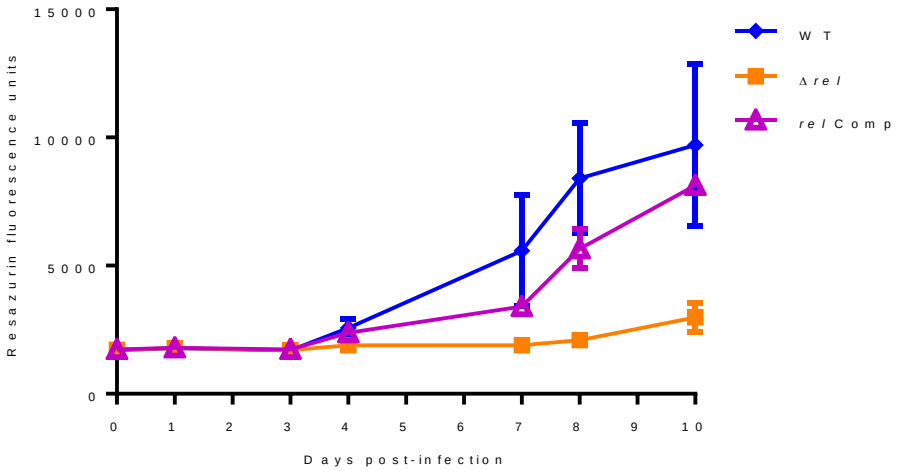

Fig. S1. Δ*rel* shows reduced growth at elevated temperature relative to its WT background strain. The cultures were grown in minimal media (7H9 + 0.1% glucose AS + 0.05% Tween-80) in a 96-well format for 10 days at 39º C. The standard resazurin assay was performed at 39º C. The data are representative of three independent experiments. RFU, resazurin fluorescence units.

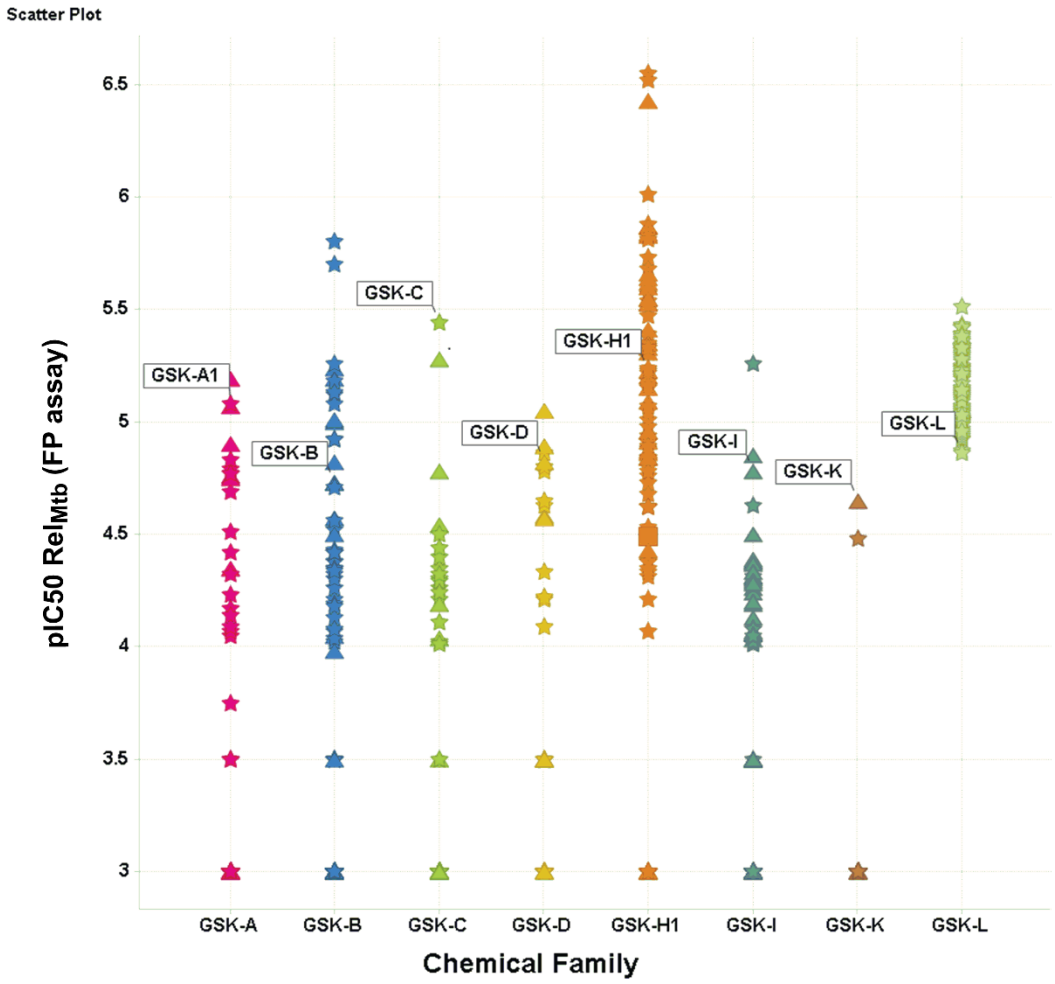

Fig. S2. Potent compounds identified by analog search of HTS hits. As a first step in the hit-to-lead process, ~1000 analogs of the 8 most promising hits (GSK-A, GSK-B, GSK-C, GSK-D, GSK-H1, GSK-I, GSK-K and GSK-M) from the HTS were found in searches carried out in corporate databases. These were tested in the Rel_{Mtb} enzymatic fluorescence polarization (FP) and HepG2 cytotoxicity assays, yielding the results presented here. We have marked the hit compound from each chemical series in the respective column. The cytotoxicity data are represented using the following symbols: ★ pIC50<4, ▲ pIC50>4 and ■ Not tested.

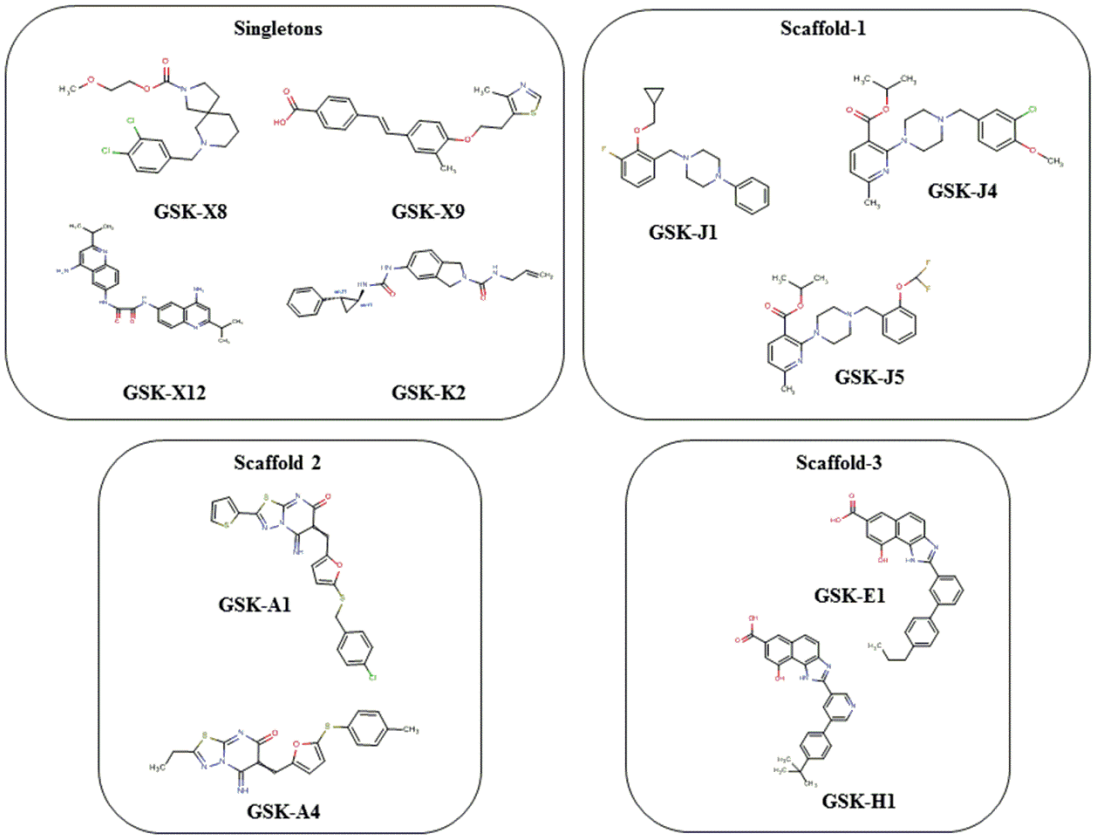

Fig. S3. Chemical structures of the 11 additional active compounds. Among these 11 hits were 4 singletons (X8, X9, X12, K2) and 3 families with more than 1 member (scaffold 1: J1, J4 and J5; scaffold 2: A1 and A4; and scaffold 3: E1 and H1).

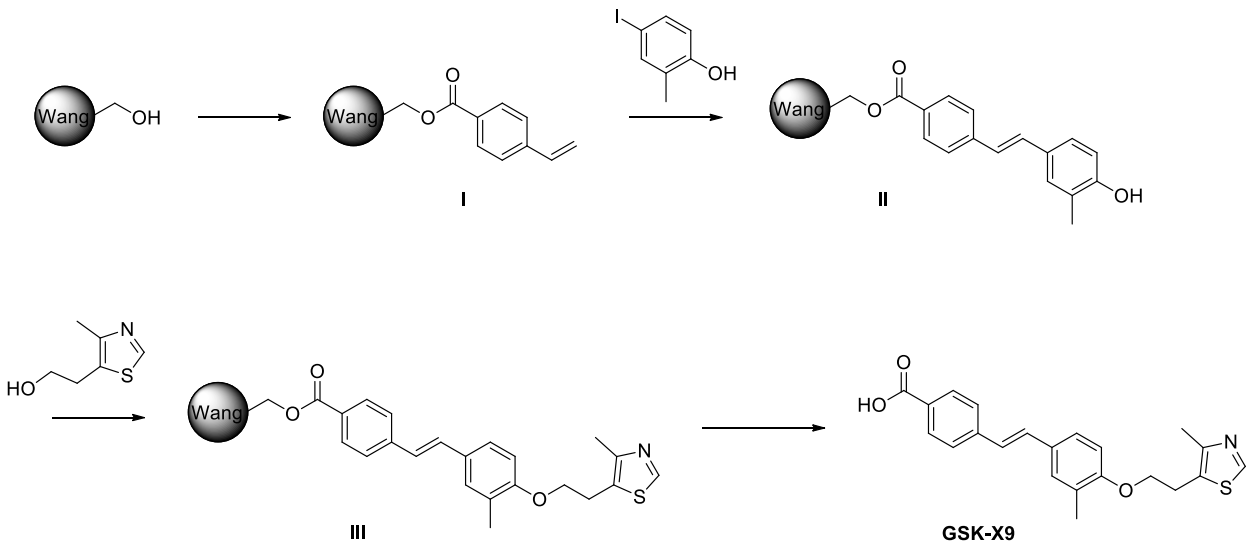

Fig. S4. Chemical synthetic route of X9.

(Synthesis of Intermediate I) To a suspension of Wang Resin (50 g, 85.0 mmol) in a 1:1 mixture of N,N-dimethylformamide: 1,2-dichloroethane at room temperature was added 4-vinylbenzoic acid (63 g, 0.425 mol, 5 eq) and 4-dimethylaminopyridine (5.2 g, 42.5 mmol, 0.5 eq). The reaction mixture was shaken at room temperature for 20 minutes. N N'-diisopropylcarbodiimide (79.9 mL, 0.510 mol, 6 eq) was then added to the reaction mixture. The reaction mixture was shaken at room temperature for 20 minutes. The reaction mixture was then placed in an incubator/shaker at 65^{o}C and shaken overnight at the same temperature. After stirring for an additional 24 hours at 60 ^{o}C the reaction mixture was cooled to room temperature and then drained. The resin was washed with N,N-dimethylformamide, then a 50% tetrahydrofuran:water mixture (x3), then alternating methanol and dichloromethane (3x each). The resin was dried. A 100 mg portion of this resin (0.17 mmol) was suspended in 1,2-Dichloroethane at room temperature. To this suspension were added N,N-diisopropylethylamine (355.4 uL, 2.04 mmol, 12 eq) and 4-dimethylaminopyridine (20.8 mg, 0.17 mmol, 1 eq). Then trifluoroacetic anhydride (236.3 µL, 1.7 mmol, 10 eq) was added. The reaction mixture was stirred at room temperature for 16 hours. The reaction mixture was drained and the resin washed with N,N-dimethylformamide (x3), water (x3), then alternating

methanol and dichloromethane (3x each). The resin was dried and sent for elemental analysis. Based on the elemental analysis (%C = 85.4, %H = 6.8, %F = 0.24) the resin loading was calculated to be 1.36 mmol/g. The product was taken on without any further purification. **(Synthesis of Intermediate II)** To a suspension of (Intermediate I) Wang-4-vinyl benzoic acid (70.0 mg, 0.095mmol) in N,N'-dimethylformamide at room temperature was added 4-iodo-2-methylphenol (178 mg, 0.952 mmol, 10 eq). Triethylamine (132.7 µL, 0.952 mmol, 10 eq) and tri-(o-toluyl)phosphine (29.0 mg, 0.095 mmol, 1eq) were added to the reaction mixture and then $Pd_2(dba)_3$ (45.3 mg, 0.048 mmol, 0.5 eq) was added. The reaction mixture was capped and then placed in the incubator/shaker at 60^{o}C and shaken at the same temperature for 2 days. The reaction mixture was cooled to room temperature and drained. The resin was then washed with N,N-dimethylformamide (x2), pyridine (x3), 0.02M sodium diethythiocarbamate trihydrate in N,N-dimethylformamide (x2), 50% tetrahydrofuran:water (x2), and then alternating methanol:dichloromethane (3x each). A portion was cleaved to yield 4-(4-hydroxy-3-methylstyryl)benzoic acid LCMS m/z = 255 $[M+H]^+$. The remaining resin used without any further purification. **(Synthesis of Intermediate III and final product)** To a solution of 2-(4-methylthiazol-5-yl)ethanol (136.3 mg, 0.95 mmol, 10 eq) in tetrahydrofuran under argon at room temperature was added triphenylphosphine (124.9 mg, 0.48 mmol, 5 eq) followed by diisopropylazodicarboxylate (93.7 µL, 0.48 mmol, 5 eq). This mixture was then stirred at room temperature for 1 hour. Wang-4-(4-hydroxy-3-methylstyryl)benzoic acid (70 mg, 0.095 mmol) was suspended in this solution at room temperature. The suspension was then warmed to 50^{o}C and shaken in the incubator/shaker at the same temperature for 20 hours. The reaction was then cooled to room temperature and drained. The resin was then washed with N,N-dimethylformamide (x4), then a 50% tetrahydrofuran:water mixture (x3), then alternating methanol and dichloromethane (3x each). The resin was then dried. The resin was cleaved with 50% trifluoroacetic acid:1,2-dichloroethane. The isolated material was purified by reverse phase HPLC (Gilson, acetonitrile:water-TFA modifier) to yield 4-(3-methyl-4-(2-(4-methylthiazol-5-yl)ethoxy)styryl)benzoic acid LCMS m/z = 255 $[M+H]^+$.

SUPPLEMENTARY METHOD TEXT: A detailed description of the high-throughput compound screen

Preparation of assay-ready plates

The screening library consisted of *circa* 2 million unique compounds with a minimum of 90% purity. Assay-ready plates were prepared by dispensing 60 nL of compound solutions in DMSO (1mM for single-concentration screening or 11-point serial dilutions down from 10mM in 1/3 steps) into black 1536-well flat bottom polystyrene plates (Greiner Bio-One Ltd., Stonehouse, UK) using an Echo 555 acoustic dispenser (Labcyte, Sunnyvale, CA). This gave a final compound concentration of 10 μM in a 6 μL final assay volume. In order to enable robust data normalization, each screening plate contained 64 negative (columns 11 and 12) and 64 positive (columns 35 and 36) control wells, which received 60 nL of DMSO (1% v/v final concentration).

HPLC-based assay of enzyme activity

This assay was based on the incubation of *Mtb* Rel_{Mtb} with adenosine 5'-triphosphate (ATP) and guanosine 5'-triphosphate (GTP), following the subsequent formation of adenosine 5'-monophosphate (AMP) by reverse-phase HPLC using ultraviolet absorbance detection. Separations were accomplished on a Zorbax Eclipse XDB C18 (4.6 x 150 mm, 5 μm) (Agilent) with a C18 Eclipse pre-column (12.5 x 4.6 mm, 5 μm) (Agilent). The mobile phase was delivered at a constant flow rate of 1.0 mL/min and the eluent was monitored continuously using a diode array detector set at 254 nm. The reaction mixture was eluted with potassium phosphate buffer (50 mM at pH 6) for 2 min; then the mobile phase gradient was increased linearly up to 12.5% methanol over 8 min and maintained at 12.5% methanol for 2 min. This method provided good chromatographic resolution and gave retention times of 1.9 min, 3.4 min, and 6.2 min for GTP, ATP, and AMP, respectively. The lower limit of quantification based on peak area was 5 μM for AMP (r^2= 0.99).

Reactions were run at room temperature in a 200-µL volume, which contained a mixture of substrates and enzyme, in a 50 mM Tris buffer (pH 8.5) containing $MgCl_2$ at half-substrate concentration, 0.5 mM DTT and 1.5 mM deoxycholic acid. After incubation at room temperature, the reaction was terminated by addition of EDTA. Then, the solution was centrifuged (3500 rpm, 2 min at 4 ºC) and a 20 µL aliquot of the sample was injected for analysis.

The apparent K_M was determined by measuring the initial rate of reaction using 200 nM of Rel_{Mtb} and determining AMP release as a function of substrate concentration ranging from 75 µM to 10 mM. The substrate concentration was varied in the presence of 10 mM of the second substrate, keeping the $MgCl_2$ at half of the concentration of substrates. The assay was incubated at room temperature (23°C) for 2 hr, and stopped at different time intervals by addition of EDTA. The initial rates of the reaction (µM AMP/min) were measured from the slopes of the linear portions of the reaction progress curves. The values of the apparent K_M and V_{max} were generated by nonlinear fit to the Michaelis-Menten equation using Grafit 7.0.2 (Erithacus Software Ltd., Horley Surrey, UK).

The enzyme titration was performed using the substrates at their K_M values, using various concentrations of recombinant Rel_{Mtb} ranging 0 to 800 nM. The assay was incubated at room temperature for 2 hr, and stopped at different time intervals by addition of EDTA. Reaction progress was followed by measuring the amount of ATP consumed and AMP released. The results indicated that the reaction is linear for 120 min with concentrations of Rel_{Mtb} up to 350 nM.

Enzyme inhibition screen

Buffer content plays a critical role in assay robustness. We optimized buffer source and pH (6.5-9.0) and also the effect of ionic strength, divalent cations, reducing agents, bovine serum albumin, detergents and polyols. When performing enzyme titration and progression curves, enzyme concentration was varied

from 0 nM to 320 nM in the presence of 1.5 mM ATP, 0.5 mM GTP and 1 mM $MgCl_2$. The reaction was stopped by EDTA addition at different times (0-120 min) at room temperature.

Before assay miniaturization, assays were performed in 384-well plates with 10 µL as final reaction volume using 1.5 mM ATP, 0.5 mM GTP, 1 mM $MgCl_2$, 4.5 nM AMP-$Alexa_{633}$ and 40 nM *M. tuberculosis* RelMtb in 50 mM Tris (pH 8.5) containing 0.5 mM DTT and 1.5 mM deoxycholic acid. Assays were generally run for 90 min at room temperature (approximately 23°C) before adding 5 µL of 45 µg/mL antibody in 50 mM Tris (pH 8.5) containing 60 µM Tween-20 and 3 mM EDTA, incubating at least 1 hr at room temperature and reading fluorescence polarization on an EnVision multilabel plate reader. Conditions for the secondary, artifact-detecting assay were the same as those described above with the exception that recombinant Rel_{Mtb} was replaced by 4 µM AMP, simulating the product typically generated by Rel_{Mtb}-catalyzed reactions in the primary assay.

Since compounds used in the HTS were dissolved in DMSO, we next determined the assay sensitivity to DMSO; DMSO up to 5% v/v did not inhibit the assay components.

Conditions for the HTS assay were the same as those described above, but the assay volume was reduced to 6 µL. Assays were performed in 1536-well black polystyrene plates by initially dispensing 3 µL of a 2× enzyme solution (80 nM Rel_{Mtb} in 50 mM Tris pH 8.5, 1 mM DTT, 3 mM Deoxycholic) into wells containing 60 nL of 1mM test compounds in DMSO. Reactions were initiated by addition of 3 µL of 2× substrate solution (3 mM ATP, 1 mM GTP, 2 mM $MgCl_2$, 6 nM AMP-$Alexa_{633}$ in 50 mM Tris pH 8.5) and then incubated (6µL reaction volume, 10µM compounds, 1% v/v DMSO) for 90 min at room temperature before addition of 3 µL of 3× detection solution (45 µg/mL antibody in 50 mM Tris pH 8.5, 60 µM Tween-20 and 3 mM EDTA). Plates were then incubated for at least 1 h before reading the fluorescence polarization using an EnVision. All additions were made using a Multidrop Combi dispenser (Thermo Fisher Scientific).

Data Analysis and Hit Scoring

The readouts from the EnVision™ multilabel plate reader were integrated with an automated HTS data management system developed internally. This system enables data analysis and quality control monitoring of the HTS campaign in real-time. For each plate the parallel fluorescence intensity ("s": Ex 620 nm, Em 688 nm) and the perpendicular fluorescence intensity ("p": Ex 620 nm, Em 688 nm) measurements were generated as readout. Fluorescence polarization (in mP) is calculated as: FP (mP) = 1000 x [(s - (G x p)) / (s + (G x p))] where "G" is the so-called "G-factor". In fluorescence polarization assays G-factor is used to correct for the bias in polarization values caused by differences in instruments and assay conditions.

The positive control signal included both substrates and enzyme (n=64, columns 11&12) and the negative control reactions lacked enzyme (n=64, columns 35&36) were used to calculate Z′ values during the screen to monitor assay robustness. The equation for Z′ compares the standard deviation of positive control (σ_+) and negative control (σ_-) to the difference in the averages of positive and negative controls (μ_+ and μ_-, respectively)

$$Z' = \{1 - [3 \times [(\sigma_+ + \sigma_-) / \left|(\mu_+ - \mu_-)\right|]\}$$

The calculated Z′ factor greater 0.5, indicating that the assay is suitable for HTS. Therefore, this miniaturized screening assay is suitable for the identification of small-molecule inhibitors of Rel_{Mtb}. Initially, compounds that altered the overall fluorescence intensity by ≥30% compared with control wells were considered either enhancers or quenchers and were excluded from further analysis. The remaining compounds were evaluated for percent inhibition, which was calculated relative to the assay plate control wells, where

$$\% \text{ inhibition} = \{100 - 100 \times [(mP_{Comp} - mP_{NegativeControls}) / [(mP_{PositiveControls} - mP_{NegativeControls})]\}$$

The results from the high throughput screening were further analyzed using using Activity Base (ID Business Solutions Ltd., Surrey, UK); a software package designed to automate the analysis of HTS data to evaluate and score different parameters and identify the most promising compounds from HTS. Patterns in the distributions of responses in the plates were detected and fixed with an in-house pattern correction algorithm (*72*).

Consistent with a typical HTS, most compounds were inactive with a distribution of % inhibition values centered on 1.8%. With an average significant statistical inhibition of 24.7% (3 × SD) as threshold (plates were analyzed in daily batches), 12,526 compounds were considered primary hits (0.63% hit rate) and selected for follow-on assays.

To determine inhibitor IC_{50} values, % inhibition of the CRCs was fitted to the standard single-site four-parameter logistic equation. For each test compound, percent inhibition was plotted against compound concentration. To calculate the IC_{50}, the data were fit to equation
$y = \{(Y_{max} – Y_{min})/[1 + ([I] / IC_{50})^{\wedge}n]\} + Y_{min}$, where Y_{max} is the maximum response, Y_{min} is the baseline, [I] is the concentration of compound, n is the Hill slope, IC_{50} is the inflection point concentration. In cases where the highest concentration tested (i.e., 100 μM) did not result in greater than 50% inhibition, the IC_{50} was determined as greater than 100 μM. $pIC_{50} = -\log IC_{50}$, expressing the IC_{50} in molar units.

HepG2 cytotoxicity assay

Actively growing HepG2 cells were removed from a T-175 TC flask using 5 mL Eagle's MEM (containing 10 % FBS, 1 % NEAA, 1 % penicillin/streptomycin) and dispersed in the medium by repeated pipetting. Seeding density was checked to ensure that new mono-layers were not more than 50% confluent at the time of harvesting. Cell suspension was added to 500 mL of the same medium at a final density of 1.2×10^5 cells/mL. This cell suspension was dispensed (25 μL, 3000 cells per well) into 384-well clear-bottom plates using a Multidrop Combi dispenser. Prior to addition of the cell suspension, the

screening compounds (250 nL) were pre-dispensed into the plates with an Echo® liquid handler. Plates were incubated for 48 hr at 37°C, 5% CO_2. After incubation, plates equilibrated at room temperature for 30 min before proceeding to develop the luminescent signal. The signal developer, CellTiter-Glo® Reagent, was allowed to equilibrate at room temperature for 30 min and added to the plates (25 μL per well) using a Multidrop Combi dispenser. Plates were left for 10 min at room temperature for stabilization and then read using a ViewLux.