Development of an intracellular screen for new compounds able to inhibit *Mycobacterium tuberculosis* growth in human macrophages

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Supplementary material

MATERIALS AND METHODS

Mycobacterial strains and plasmid construction. Mtb H37Rv (*ATCC* 25618) and Mtb Erdman (*ATCC* 35801) were utilized for all experiments. Both strains were routinely grown at 37° C in 7H9 medium supplemented with 0.2% glycerol, Middlebrook ADC enrichment (0.5% bovine albumin fraction, 0.2% dextrose, 0.003% catalase), 0.05% tyloxopol. When required, hygromycin B was added to the medium at a final concentration of 50 μg ml^{-1}.

Molecular biology techniques were carried out according to standard procedures (1). The acetamide-inducible luciferase assay vector pATB45Luc was obtained using pATB45 as a backbone, a mycobacterial shuttle plasmid derived from pACE-1, containing an acetamidase promoter (2) upstream of a multi-cloning site and a hygromycin marker. The *luc* gene, encoding luciferase from *Photinus pyralis* (GenBank Accession Number M15077) was double digested with *XhoI* and *BamHI*. The derived 1716 bp-long fragment was subsequently blunt-ended using T4 DNA Polymerase

(Invitrogen). This molecule was ligated into a pATB45 vector, previously linearized by *ScaI* digestion and dephosphorylated with Shrimp Alkaline phosphatase (Roche). The final plasmid, pATB45Luc, was transformed in *E.coli* DH5α and recombinant strains were verified by restriction analysis and by colony PCR. The strain Mtb H37Rv pATB45Luc was made by transforming competent WT *Mtb H37Rv* with plasmid pATB45Luc and plating on selective media.

The strain Mtb Erdman GM-T-15 GFP_{des} was a kind gift from Dr Neeraj Dahr. This is a GFP-reporter strain constructed by inserting a destabilized GFP-coding sequence upstream of the *rrnA* operon into the mycobacterial chromosome (3).

Cell culture. The human monocytic cell line THP-1 (ATCC TIB-202) was used in this study. THP-1 cells were grown and maintained in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma) and 1 mM sodium pyruvate (Sigma). Cells were monitored using an optical microscope (Nikon), counted with a NucleoCounter NC-100 (Chemometec) and maintained between 2 and 10×10^5 cells ml^{-1} at 37° C in a humidified, 5% CO_2 atmosphere. THP-1 cells were treated according to GSK policies for management of human biological samples.

Compounds. Compounds in dimethyl sulfoxide (DMSO) were robotically dispensed into white or black-walled, clear-bottom 384-well plates, for primary and secondary assay, respectively, with a final DMSO concentration of 0.5%. For single concentration experiment, the final compound concentration was 50 μM. For potency determinations, eleven-point one in three dilution curves were generated with a top concentration of 50 μM.

Preparation of Mtb single cell suspensions. A single cell suspension of Mtb H37Rv pATB45luc and Mtb Erdman GM-T-15 GFP_{des} were prepared prior to infection. 25 ml of bacterial culture grown to log phase was centrifuged at 2860*g* for 10 min. After removal of the supernatant, cell clumps were dispersed by vigorously shaking with sterile glass beads (Sigma) for 2 min. Dispersed cells were then re-suspended in 10 ml of RPMI medium and left to decant for 5 min at room temperature. Cells were then centrifuged at 402*g* for 5 min. Supernatant was collected and its OD600 was measured. OD ml^{-1} was converted to CFU ml^{-1} considering that 0.125 OD is equal to approximately 10^7 CFU ml^{-1}.

Infection of THP-1 cells with Mtb and incubation with compounds. 3 x 10^8 cells of THP-1 cells were simultaneously differentiated with phorbol myristate acetate (PMA, 40 ng ml^{-1}, Sigma) and infected with a single cell suspension of Mtb H37Rv-pATB45luc (primary assay) or Mtb Erdman GM-T-15 GFP_{des} (secondary assay) in a roller bottle at a MOI of 0.5:1, 1:1, 5:1 or 10:1. Cells were put in a roller bottle apparatus for 4 hours at 37° C at 1.5 rpm. After incubation, infected cells were washed four times by centrifugation at 400 *g* for 5 min and re-suspended in fresh RPMI medium to remove extracellular bacilli. In the last wash, infected cells were re-suspended in RPMI medium supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine and pyruvate at a concentration of 2 x 10^5 cells ml^{-1}. 50 µl of this cell suspension (typically 10000 cells per well) were dispensed into the wells of 384-well plates (white, flat bottom, Greiner, for primary assay and CellCarrier-384 collagen-coated, black, optically clear glass bottom, PerkinElmer, for secondary assay). Prior to addition of the cell suspension, the screening compounds (250 nL) were dispensed into the plates with an Echo liquid handler. Plates were allowed to incubate at 37° C at 80% relative humidity for 3, 4 or 5 days under 5% CO_2.

Primary assay with luciferase reporter. Luciferase activity, proportional to bacterial load, was determined by using the BrightGlo™ Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Resultant luminescence was measured in an Envision Multilabel Plate Reader (PerkinElmer) using the 384-plate Ultra Sensitive luminescence mode, with a measurement time of 200 ms per well.

Secondary assay with GFP reporter. GFP concentration was measured in single bacterial cells by image analysis. Infected macrophages were fixed by a formaldehyde solution (final concentration 4% v/v) and subsequent incubation at room temperature for 30 min. Formaldehyde solution was then removed and cells were washed twice with 50 µl of Hank´s Balanced Salt Solution (HBSS buffer, GIBCO). Nuclei of the THP-1 cells were stained by the addition of DRAQ5 far-red fluorescent DNA dye (BioStatus, 5 µM final concentration) in the dark. After 1 hour incubation at room temperature, plates were sealed with optical adhesive film and parafilm and decontaminated with T.B.Q.® detergent (Steris) prior to removal from Biosafety Level 3 lab. Cells were observed and imaged using an Opera High Content Screening System (PerkinElmer) and 20x water immersion objective with 488-nm (green, GFP) and 635-nm (red, DRAQ5) excitation wavelengths. Six images per well were collected for reliable statistical analysis. Automated image analysis was performed with a script developed on Acapella High Content Imaging and Analysis Software (PerkinElmer). Different parameters were measured such as number of cells, area, size, roundness, signal intensity, spots, spot classification, spot intensity and texture properties. THP-1 cell count, bacterial load per macrophage and percent of infected cells were reported for each well corresponding to a specific compound concentration.

Data analysis. Every assay plate contained a column of negative controls with DMSO, which correspond to 100% bacterial growth, and a column of positive controls in which

100% inhibition of bacterial growth was reached by adding 5 μM rifampicin. These controls were used to monitor assay quality through determination of Z' as well as for normalizing the data on a per-plate basis. The Z′factor was determined using the formula:

$$Z' = 1 - \frac{3\,(\mathbf{SD}\text{rifampicin} + \mathbf{SD}\text{DMSO})}{|\mathbf{M}\text{rifampicin} - \mathbf{M}\text{DMSO}|}$$

where SD is the standard deviation and M is mean. For the primary assay, the effect of a given compound is calculated as:

$$\text{Percentage inhibition} = 100 \times \frac{\text{sample signal} - \text{DMSO signal}}{\text{rifampicin signal} - \text{DMSO signal}}$$

The minimum inhibitory concentration (MIC90) was determined as the lowest concentration of compound required to produce a >90% decrease in observed luminescence. For the secondary assay, the effect of a given compound was calculated as:

$$\text{Percentage killing} = 100 \times \frac{\text{sample signal} - \text{DMSO signal}}{\text{rifampicin signal} - \text{DMSO signal}}$$

The inhibitory concentration (IC90) was determined as the lowest concentration of compound required to decrease the bacterial load > 90% (percentage infection < 10%).

MIC90 interpolations were performed with GraphPad Prism software using a non-linear regression analysis normalized 4 parameter log dose with variable slope curve.

Supplementary Figure 1

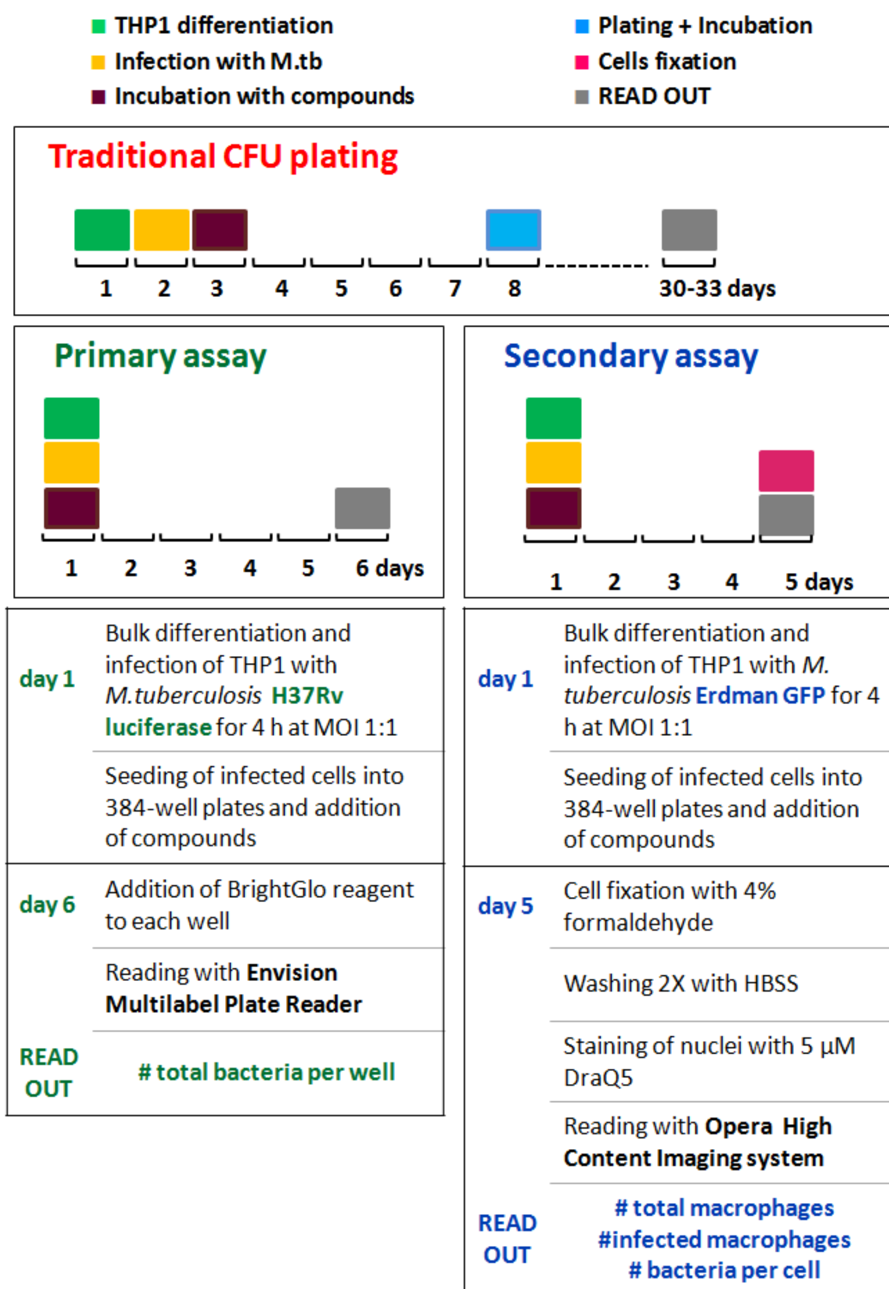

Figure S1 - Primary and secondary assay protocols for the determination of intracellular MIC in human macrophages. Intracellular bacterial count is generally achieved by the traditional CFU method. THP-1 cells are differentiated on day 1, infected on day 2 and incubated with test compounds on day 3. After 5 days of incubation with the compound, infected cells are lysed and 10-fold serial dilutions of

the lysate are plated on 7H10 agar. Colony counts are finalized after 3 weeks of incubation at 37°C. In total, the entire CFU assay takes 30-33 days to complete. The primary and secondary assays developed in this work make use of TB strains expressing reporter genes. Bacterial load can be quantified by measuring the activity of the reporter protein, thus dramatically reducing the assay times. The primary assay relies on a H37Rv strain expressing firefly luciferase, while the secondary assay utilizes an Erdman strain expressing unstable GFP. In the protocol developed, differentiation and infection of THP-1 cells and incubation of the infected cells with the compounds to be tested were performed on the same day, further decreasing the assay time to only 6 and 5 days, for primary and secondary assay respectively. For the primary assay, BrightGlo™ reagent containing the luciferase substrate was added to each well prior to plate reading in an Envision MultiPlate reader. Final readout obtained was RLU which represent the total number of bacteria per well. For the secondary assay, cells were first fixed with formaldehyde, washed with HBSS and nuclei were stained with DraQ5. Plates were imaged with an Opera High Content System, which gave as readouts the total number of cells, total number of bacteria and total number of infected cells.

Supplementary Figure 2

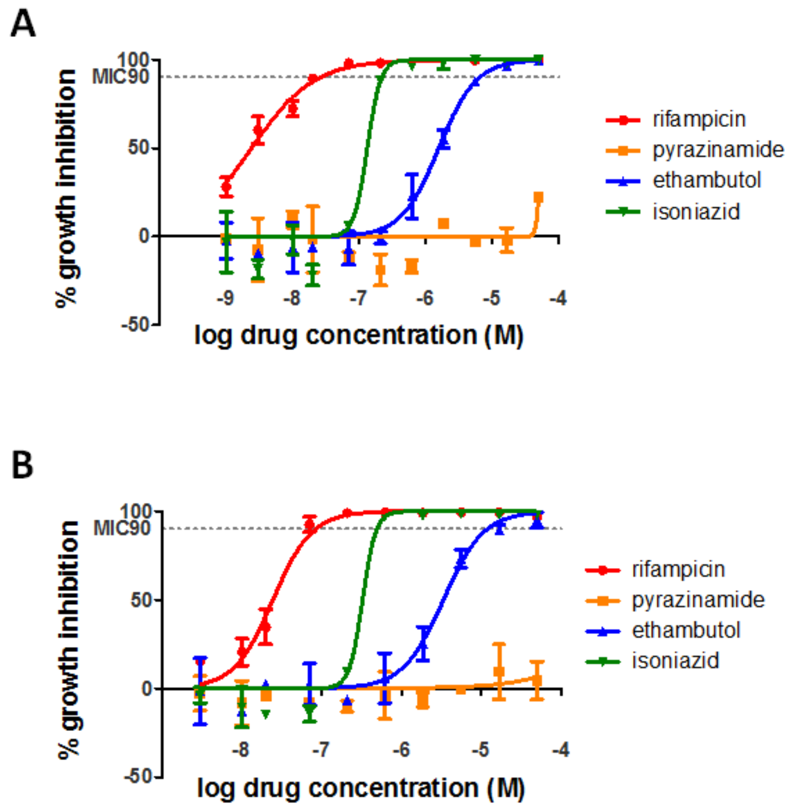

Figure S2 - Dose response tests showing percent growth inhibition using the primary assay (**A**) and percentage of killing using the secondary assay (**B**) for 4 representative TB standard drugs. Treatment with solvent alone (0.5% v/v DMSO) served as negative control with 0% inhibition of growth, while treatment with 5 µM rifampicin was used as positive control with 100% inhibition of growth. Data were normalized using negative and positive controls. MIC90 values were calculated as described in Materials and Methods. Results are representative of two independent experiments with standard deviation (SD). Non linear regression was used to fit the data of the log (inhibitor) vs. response (variable slope) curve by using GraphPad analysis software.

Supplementary Figure 3

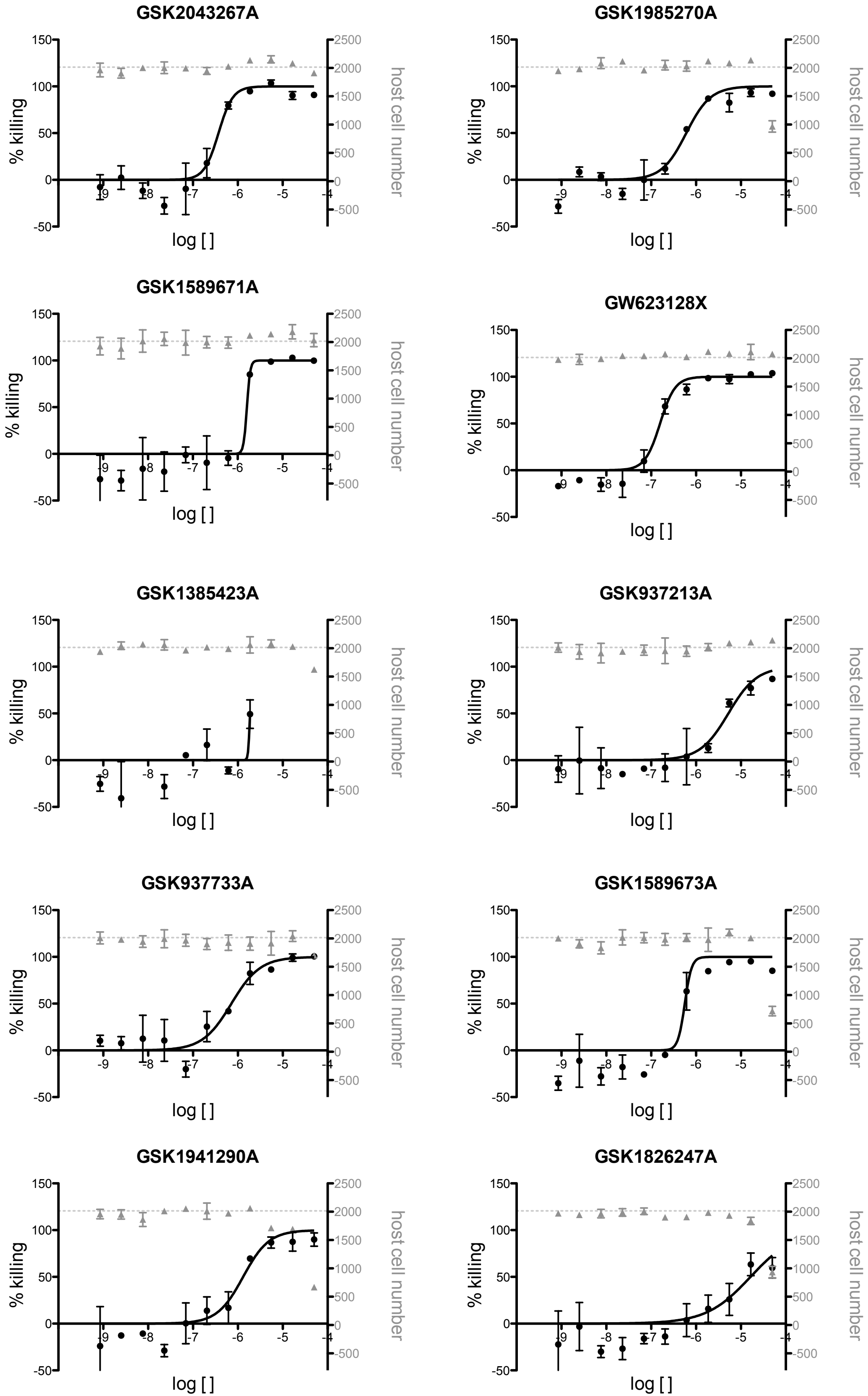

Figure S3 - Secondary intracellular assay for the top 10 compounds from the set of *in vitro* actives. Percentage of infected cells (black circles) and host cell number (gray triangles) were determined by customized image analysis. Results are representative of two independent experiments run in triplicates with standard deviation (SD).

Supplementary Figure 4

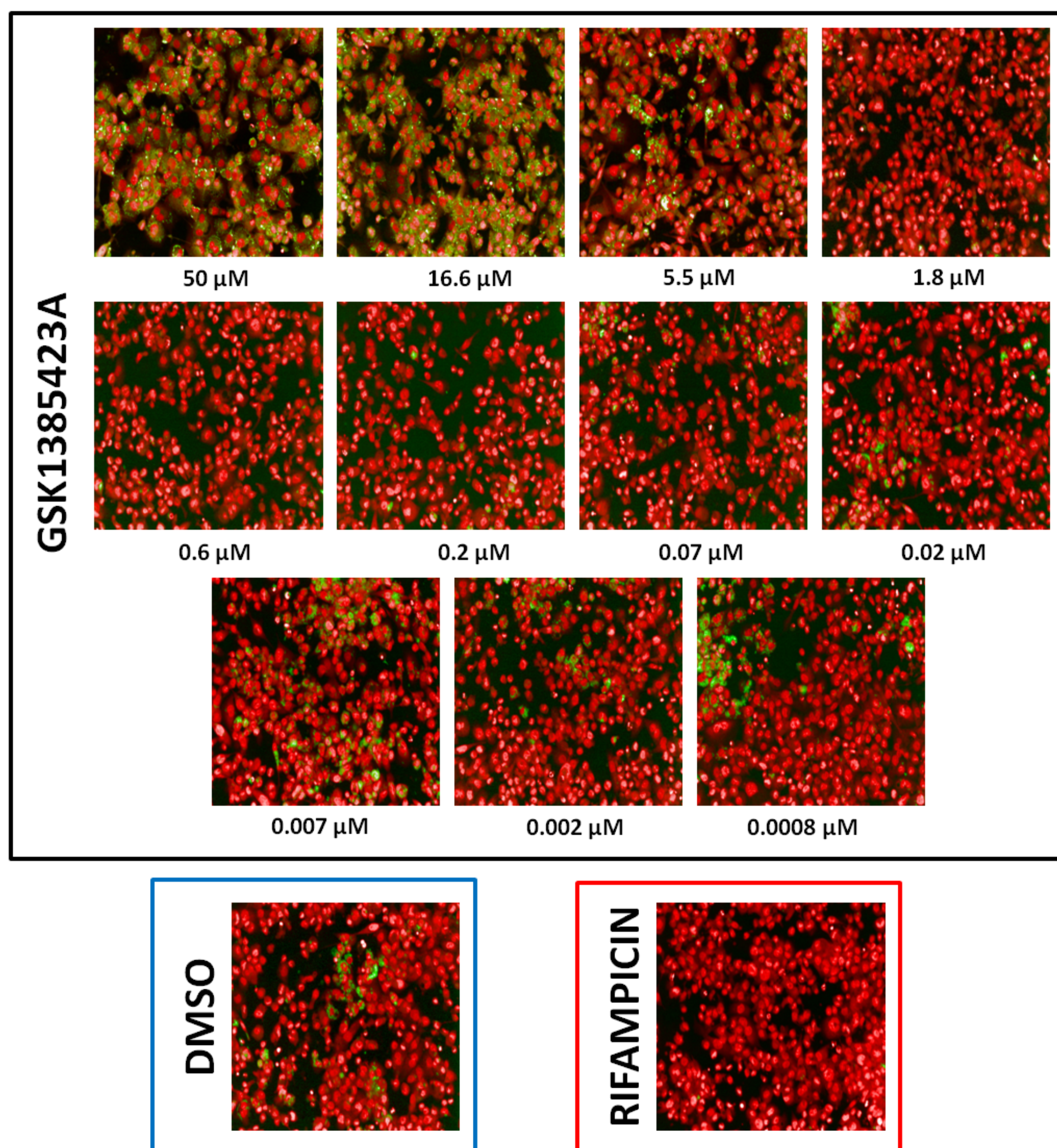

Figure S4 – High-content images of THP-1 cells infected with *Mtb* Erdman-GFP at day 4 and treated with different concentrations of compound GSK1385423A, rifampicin (5 µM) or DMSO (0.5 %) control

Table S1 – Intracellular MIC90 determined by the luciferase assay, extracellular MIC90 determined in (4) and extraMIC90/intraMIC90 fold change of the compounds belonging to the TB set.

Compound number	Intracellular MIC90 in THP-1 (µM)	MIC90 in H37Rv MIC90 (µM)	MIC90 in H37Rv /MIC90 in THP-1
GW623128X	0.07	1	14.28
GSK353069A	0.07	0.2	2.86
GSK2200150A	0.21	1	4.76
GSK2200160A	0.21	1	4.76
GSK1589671A	0.21	4.4	20.95
GSK1180781A	0.21	1.2	5.71
GSK1985270A	0.21	4.2	20
GSK3011724A	0.21	1.3	6.19
GSK1829676A	0.21	0.2	0.95
GSK2043267A	0.21	3.7	17.62
GW861072X	0.61	0.3	0.49
GSK1941290A	0.61	4.8	7.87
GSK937213A	0.61	6.1	10
GSK2032710A	0.61	4.2	6.88
GSK1589673A	0.61	4.9	8.03
GSK1829820A	0.61	0.2	0.33
GSK1829736A	0.61	0.9	1.47
GSK1826247A	0.61	4.7	7.70
GSK1385423A	0.61	8.4	13.77
GSK937733A	0.61	5.4	8.85
GSK1829819A	0.61	1.3	2.13
GSK1829729A	0.61	0.9	1.47
CCI7967	0.61	2.4	3.93
GSK381407A	0.61	4.5	7.38
GR135487X	0.61	2.2	3.60
SB-811796-V	1.85	7.8	4.22
GSK358607A	1.85	0.4	0.22
GSK345724A	1.85	5.3	2.86
GSK636544A	1.85	5.2	2.81
GSK810037A	1.85	2.4	1.30
GSK1955236A	1.85	1.5	0.81
SB-746177	1.85	9.2	4.97
GSK888636A	1.85	0.6	0.32

GSK847913A	1.85	3.3	1.78
GSK385518A	1.85	3.7	2
GSK237561A	1.85	4	2.16
GSK498315A	1.85	2.9	1.57
GSK847920A	1.85	5	2.70
GSK353071A	1.85	3.2	1.73
GSK1826089A	1.85	7.6	4.11
GSK1744926A	1.85	1.9	1.03
GSK1055950A	1.85	7.6	4.11
GSK1829732A	1.85	1.1	0.59
GSK1829728A	1.85	3.4	1.84
GSK1829727A	1.85	3.9	2.11
GSK1829733A	1.85	2.1	1.13
BRL-7940SA	1.85	2.3	1.24
GSK735826A	1.85	2.7	1.46
GR223839X	1.85	2.7	1.46
GSK735816A	1.85	2.3	1.24
BRL-10143SA	1.85	3.2	1.73
GSK731389A	1.85	1.6	0.86
GSK1857145A	5.55	6.8	1.22
GSK798463A	5.55	3.7	0.67
SB-712970	5.55	4.5	0.81
GSK1121877A	5.55	1.4	0.25
GSK1829660A	5.55	6.2	1.12
GSK762874A	5.55	3.1	0.56
GR135486X	5.55	0.2	0.04
GSK275984A	5.55	9	1.62
GSK463114A	5.55	1.1	0.20
GSK920703A	5.55	8.4	1.51
GSK1402290A	5.55	5.5	0.99
GSK2059310A	5.55	1.8	0.32
SB-435634	5.55	1.8	0.32
GSK695914A	5.55	2.2	0.40
GSK1051703A	5.55	7.1	1.28
SB-516933	5.55	3.7	0.67
GSK994258A	5.55	3.4	0.61
GSK861337A	5.55	2.7	0.49
GSK1758774A	5.55	6.6	1.19
GSK1302651A	5.55	5.9	1.06
GSK1434490A	5.55	5.2	0.94
GSK1832831A	5.55	8.3	1.49
GW369335X	5.55	8.7	1.57
GSK1731114A	5.55	5.3	0.95
GSK889423A	5.55	8.8	1.58
SB-811137-V	5.55	4.7	0.85

GW859039X	5.55	1.7	0.31
GSK957094A	5.55	2	0.36
GSK353496A	5.55	3.4	0.61
GSK892651A	5.55	9	1.62
GSK1829674A	5.55	3.1	0.56
GSK124576A	5.55	2.3	0.41
GSK749336A	5.55	0.3	0.05
BRL-51091AM	5.55	3.5	0.63
GW664700A	5.55	8.7	1.57
BRL-8088SA	5.55	2.3	0.41
GSK2111534A	5.55	0.4	0.07
GSK810016A	5.55	1.5	0.27
BRL-10988SA	16.65	2.3	0.14
GSK130506A	16.65	5.6	0.34
GSK1829671A	16.65	3.3	0.20
GW857165X	16.65	3.3	0.20
GSK124945A	16.65	2.8	0.17
GSK1518999A	16.65	9.2	0.55
SB-204804-A	16.65	1.1	0.07
GSK1859936A	16.65	2.8	0.17
GSK754716A	16.65	4.7	0.28
GSK1759150A	16.65	1.1	0.07
GSK1863309A	16.65	7.8	0.47
GSK1925843A	16.65	5.6	0.34
GSK275628A	16.65	5.2	0.31
GSK479031A	16.65	7.6	0.46
GSK1829816A	16.65	7.7	0.46
GSK848336A	16.65	7.1	0.43
BRL-8903SA	16.65	3.8	0.23
GSK921190A	16.65	1.7	0.10
GSK347301A	16.65	8.3	0.50
GSK1174628A	50	9.8	0.20
GSK276001A	50	7.4	0.15
GSK1691553A	50	7.9	0.16
GSK920684A	50	3.5	0.07
SB-829405	50	0.5	0.01
GW360240X	50	3	0.06
GSK1788487A	50	8.4	0.17
GSK352635A	50	9.1	0.18
GSK163574A	50	0.3	0.01
GSK1733953A	50	8.1	0.16
GSK1611550A	50	9.8	0.20
GSK1750922A	50	1.9	0.04
GSK146660A	50	7.9	0.16
GSK1372568A	50	8.4	0.17

GSK270670A	50	6.1	0.12
GSK1729177A	50	6.6	0.13
BRL-51093AM	50	5.3	0.11
GSK1635139A	50	9.5	0.19
GSK1072678A	50	3.7	0.07
GSK921295A	50	8.7	0.17
GV187303X	50	9.4	0.19
GSK1650514A	> 50		
GSK1783710A	> 50		
GSK1668869A	> 50		
GSK1742694A	> 50		
GSK1570606A	> 50		
GSK2157753A	> 50		
GSK316438A	> 50		
SB-650816	> 50		
GSK437009A	> 50		
GR153167X	> 50		
GSK1365028A	> 50		
GSK445886A	> 50		
GSK547511A	> 50		
GSK426032A	> 50		

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