High throughput screening (HTS) to identify ATP inhibitors on hypoxic non-replicating mycobacteria.

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Supplementary Methods

In vitro models of *M. tuberculosis* for non growing cells has been developed to test drugs that target latent TB, some of them based on a particular stress that the pathogen may encounter in the host. These include, the oxygen depletion model[1, 2], treatment with nitric oxide[3] and growth

in acidic medium[4], which mimics the hypoxic environment and stresses *M. tuberculosis* confront during infection. Recently, these treatment were combined[5] and other model of non-growing cells that exploits the streptomycin dependent *M. tuberculosis* has been reported[6]. While these models contributed to decipher aspects of *M. tuberculosis* phenotypes in dormancy they may be cumbersome for application to high throughput screening (HTS) assays and are not designed to specifically target the maintenance of proton motive force and ATP homeostasis. A reproducible and manageable model to specifically target the respiratory function of non-growing cells is therefore justified. In this study, we developed a phenotypic screen designed to identify compounds that target the process of ATP synthesis and maintenance under hypoxic conditions in *M. tuberculosis*.

In order to screen large numbers of compounds, the hypoxic shift down described in methods was miniaturized and adapted to 1,536-well format. It was estimated that 5 L of hypoxic culture were required to complete the HTS screen hence we needed to produce sufficient volume of hypoxic non-replicating culture to support a reasonable throughput. We found that preparing hypoxic shift down cultures in 400 ml/bottle using a roller-bottle setup housed inside the anaerobic chamber was an effective way to achieve large culture volumes with even gas exchange. The ATP level in large batch cultures prepared in this way was identical to that observed with the pilot experiments and showed similar nitrate dependence.

Screening 600,000 compounds in 1536 well high density format and identifying a suitable compound addition mechanism was challenging due to several constraints: (1) the compound library is stored as DMSO solution under normoxic conditions; (2) the HTS workflow design and equipment are not easily adaptable to hypoxic environment; and (3) any perturbation should not compromise compound integrity. Initial attempt to dry spot the compounds was not successful because we found that a significant proportion of compounds would either precipitate or not reconstitute (data not shown). The acoustic droplet ejection (ADE) method is highly precise and allows small volumes of compound to be efficiently transferred such that pre-dilution step (during which compounds often precipitate out of solution) or placement of excipient material (eg. media) in the assay plate are no longer necessary.

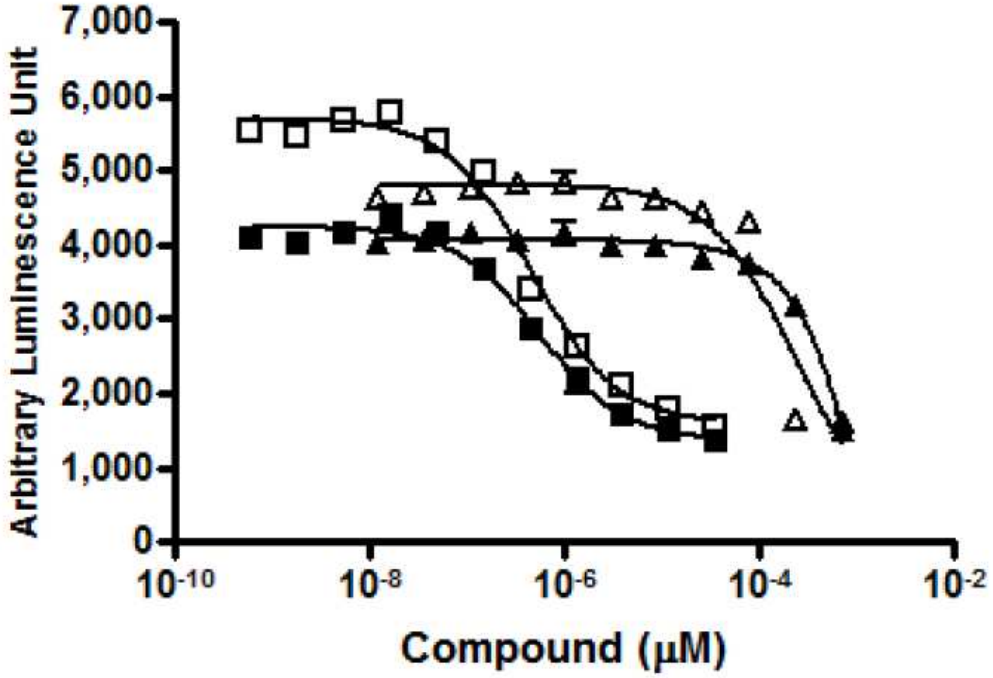
A custom copy of our library was created so that the microtiter plate type would be compatible with the ADE equipment. Over 500 “assay-ready” plates were created with 100 nl of compound as a DMSO droplet in each well. The plates were placed inside the anaerobic chamber for equilibration of 24 hours before receiving the hypoxic cells (Supplementary file Figure 1).

All compounds in the library were resuspended in DMSO solution at stock concentration of 1 mM and plated in 1536 well plate made of cyclic olefin copolymer which is compatible with the acoustic dispenser (ECHO 555). Using a no-contact transfer technology, 100 nl of each compound was transferred well by well to a dry 1536 well white solid bottom assay plate (Greiner catalog# 789173A). The assay plates were then stored in the anaerobic chamber and used within 48 hours in an experiment.

HTS and initial hit confirmation were performed using *M. bovis* BCG_{MtbNar} strain. To carry out hypoxic shift down assay mycobacteria using large volume of culture, a roller-bottle platform was placed inside an anaerobic chamber set at 37°C such that multiple bottles of culture (400 ml/bottle) could be incubated simultaneously. Similar to the process employed for the 24 well assay described above, a mid-log aerobic culture was washed and resuspended to OD_{600} of 0.2 before introduction into the anaerobic chamber for the hypoxic shift down process. Once the downshifted cultures received the additional 20 mM nitrate as described above it was ready for compound challenge. We placed a Deerac Equator liquid dispenser in a conjoined anaerobic chamber. Without removing the culture from the hypoxic environment, the culture was dispensed at 7µl per well into assay-ready plates prepared as described above such that the final screening concentration is 14 µM. Each assay plate was then covered with a metal lid with air hole for gas exchange (GNF systems). To minimize evaporation, the lidded plates were placed inside a zipped plastic bag in groups of 6, and further incubated at 37°C for 48 h. Deerac Equator liquid dispenser dispensed 2.5 µl per well of BacTiter-Glo into the microtiterplates. Every 6 plates were then removed from the anaerobic chamber and incubated at ambient environment for 15 minutes to allow luminescence signal development prior to reading on a CCD-camera based plate reader (CLIPR by Molecular Devices) for 20 sec exposure per plate. The above procedures were performed on batches of 80 assay plates, such that the screening campaign of 600,000 compounds was completed in 4 weeks. Luminescence signal was normalized on a per plate basis with median signal set as 1.0. Compounds with activity of less than 0.7 were scored as a hit.

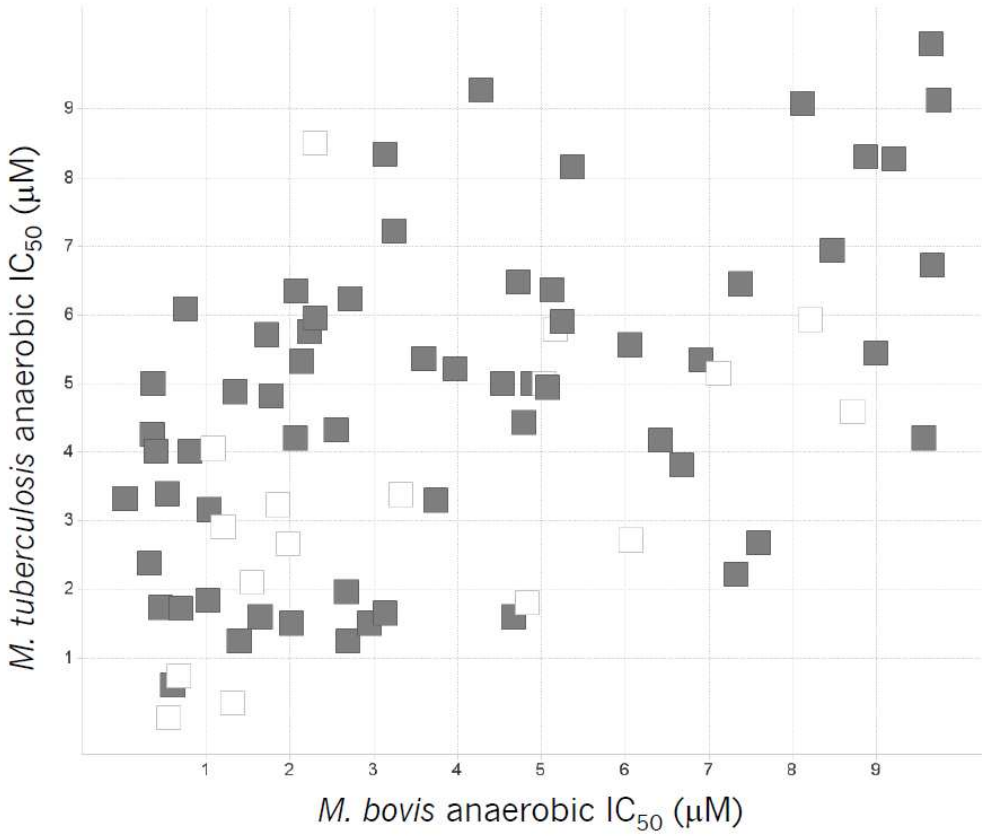
Anaerobic chamber maintenance. Anaerobic chambers were purchased from Coy Lab, and establishment of the hypoxic atmosphere was done as per manufacturer's instructions. During routine operation, the palladium catalyst was renewed every week and the hydrogen level within the chamber was maintained at 3-4% to ensure that any trace of oxygen was effectively removed. Oxygen sensor monitoring indicated that the atmosphere had less than 20 ppm at all times.

Supplementary figures with legends

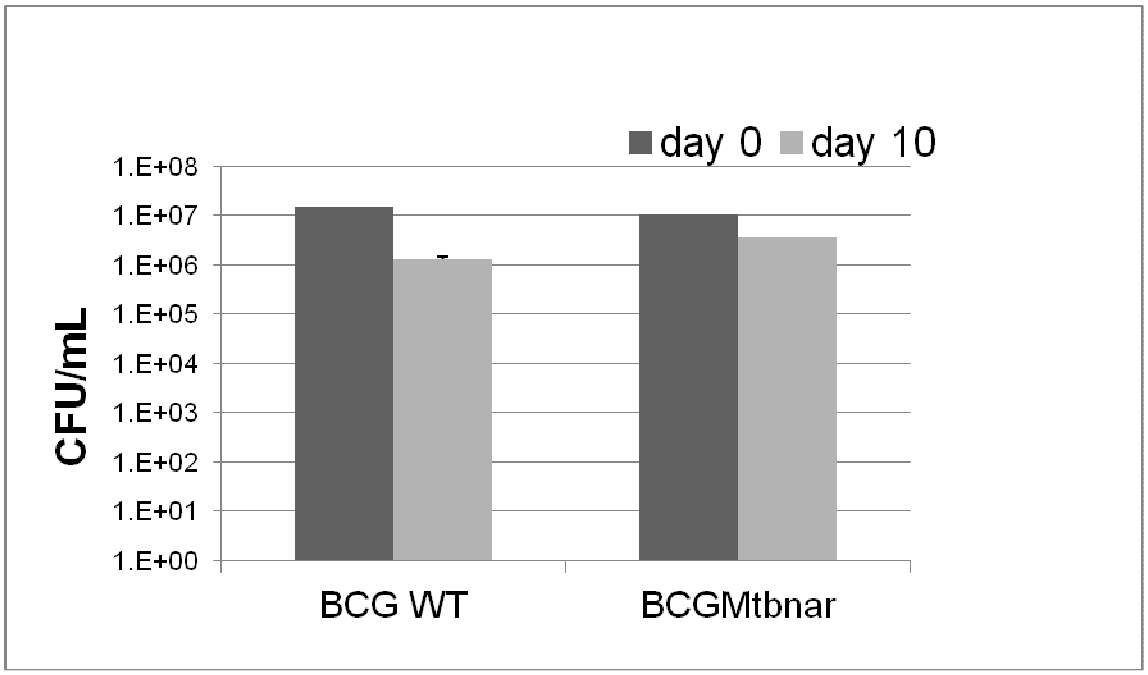


Supplementary figure 1. Compounds dispensed as DMSO droplet required 24 hrs pre-equilibration in hypoxic condition.

TMC207 (square) and DCCD (triangle) stored under normoxic condition were dispensed as 50 nl droplets either on the day prior (solid) or freshly (open) onto assay plates. The assay was performed in the form of 11 points dose response in duplicates. The plates were then introduced into anaerobic chamber for the experiment with hypoxic downshifted *M. bovis* BCG_{MtbNar} for 48 hr of compound treatment.



Supplementary figure 2. Comparative analysis of hypoxic activities between *M. bovis* BCG_{MtbNar} and *M. tuberculosis*. Actives compounds identified in the hypoxic shift down *M. bovis* BCG_{MtbNar} assay were tested in hypoxic shift down *M. tuberculosis*. ATP levels (RLU) were quantified by using the BTG Assay Kit. The assay was performed in the form of 8 points dose response, starting at the top dose of 10 µM in duplicates. Data show the distribution of the hypoxic ATP IC_{50} activity in both strains after two days of drug exposure. Grey box represent hits with only hypoxic ATP IC_{50}. White box represent hits with aerobic MIC_{50} and hypoxic ATP IC_{50} activity.



Supplementary figure 3. Survival of *M. bovis* BCG and *M. bovis* BCG_{Mtbnar} in the hypoxic shift down model. Viability (CFU/ml) of *M. bovis* BCG parental and *M. bovis* BCG_{Mtbnar} strains was determined at 0 (black bars) and 10 days (gray bars) of the hypoxic shift down model. The number of viable cells was determined by CFU counts on 7H11 agar. Results are expressed as the means ± SD of triplicates.

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