Published in final edited form as:

ACS Chem Biol. 2013 October 18; 8(10): . doi:10.1021/cb4004817.

Identification of novel inhibitors of non-replicating *Mycobacterium tuberculosis* using a carbon starvation model

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Abstract

During *Mycobacterium tuberculosis* infection, a population of bacteria is thought to exist in a non-replicating state, refractory to antibiotics, which may contribute to the need for prolonged antibiotic therapy. The identification of inhibitors of the non-replicating state provides tools that can be used to probe this hypothesis and the physiology of this state. The development of such inhibitors also has the potential to shorten the duration of antibiotic therapy required. Here we describe the development of a novel non-replicating assay amenable to high-throughput chemical screening coupled with secondary assays that use carbon starvation as the *in vitro* model. Together these assays identify compounds with activity against replicating and non-replicating *M. tuberculosis* as well as compounds that inhibit the transition from non-replicating to replicating stages of growth. Using these assays we successfully screened over 300,000 compounds and identified 786 inhibitors of non-replicating *M. tuberculosis*. In order to understand the relationship among different non-replicating models, we tested 52 of these molecules in a hypoxia model and four different chemical scaffolds in a stochastic persist model and a streptomycin dependent model. We found that compounds display varying levels of activity in different models for the non-replicating state, suggesting important differences in bacterial physiology between models. Therefore, chemical tools identified in this assay may be useful for determining the relevance of different non-replicating *in vitro* models to *in vivo* *M. tuberculosis* infection. Given our current limited understanding, molecules that are active across multiple models may represent more promising candidates for further development.

Introduction

One third of the world's population is infected with *M. tuberculosis*, with 1.4 million tuberculosis-related deaths and 8.8 million new infections occurring each year (1). Successful treatment of active, symptomatic, tuberculosis infection requires a complex regimen over at least six months. Treating clinically asymptomatic or latent infection in which the host immune system contains *M. tuberculosis* growth but does not eradicate infection, requires multi-drug therapy for 3 months or mono-therapy for as long as 9 months (2).

During both active and latent infection, a subpopulation of *M. tuberculosis* bacteria is thought to exist in a non-replicating, metabolically less active state, refractory to many antibiotics (3, 4). The term phenotypic antibiotic tolerance is used to describe the reduced efficacy of antibiotics against non-replicating bacteria in the absence of genotypic resistance (5-7). The presence of non-replicating, phenotypically tolerant bacteria within the host could contribute to the need for prolonged drug therapy for active and latent tuberculosis infection (8, 9).

Several *in vitro* models for non-replicating *M. tuberculosis* have been proposed. In an environmental model, exposing bacteria to a microenvironment likely encountered during the course of infection, such as oxygen limitation, nutrient deprivation or low pH, results in a non-replicating and antibiotic tolerant state (10-14). Alternatively, a stochastic model has been proposed in which a small subpopulation of antibiotic tolerant bacteria, often called persisters, exists within a larger genetically identical antibiotic-susceptible bacterial population (15). While studies disagree on the growth rate of this subpopulation, the antibiotic tolerant cells can be identified by their survival after a culture is exposed to high doses of antibiotics (16-20). Most recently, a *M. tuberculosis* mutant strain that requires streptomycin for growth was suggested as an additional non-replicating model. In the absence of streptomycin the bacteria stop replicating and exhibit tolerance to antimicrobials (21, 22).

While there is no consensus on which *in vitro* non-replicating model is the most relevant to *in vivo* infection, several observations support the relevance of the nutrient deprivation model. *M. tuberculosis* isolated directly from lung lesions has an altered morphology and reduced acid-fast staining similar to *M. tuberculosis* starved in phosphate-buffered saline (PBS) *in vitro* (23). Furthermore, when the enzyme Rel_{MTB}, which mediates adaptation to nutrient deprivation by the formation of hyperphosphorylated guanine nucleotides (ppGpp), is deleted, the bacteria are significantly impaired in their ability to establish persistent infection in mice and guinea pigs (23-25).

The majority of current anti-tubercular drugs used clinically act on molecular targets essential for active cellular replication, including cell wall, DNA and RNA biosynthesis, and are ineffective against non-replicating bacteria (26). Two notable exceptions include bedaquiline (TMC207), the recently approved FDA drug for drug resistant TB that targets ATP synthase, and clofazamine, an antibiotic approved for the treatment of leprosy that targets the enzyme NDH-2 within the electron transport chain (27). Recent studies comparing the efficacy of 3- and 4-drug combinations including bedaquiline and clofazamine in a murine model of tuberculosis suggest that regimens containing these agents are superior to the standard first-line regimen of rifampin (RIF), pyrazinamide and isoniazid (INH) (28). In addition, the small molecule PA-824, currently in phase 2 clinical trials, also exhibits bactericidal activity against non-replicating bacilli (29), and when given in combination with moxifloxacin and pyrazinamide, exhibits promising sterilizing activity in murine models of tuberculosis (30). Understanding the physiology of non-replicating bacteria and the relevance of the different *in vitro* models to *in vivo* infection would provide insight into the discovery of more effective therapeutics. Furthermore, novel inhibitors of processes essential for the survival of non-replicating bacteria have the potential to shorten the duration of antibiotic therapy for both active and latent tuberculosis infection.

Recently several approaches have been used to identify compounds focusing on non-replicating activity, including targeted and phenotypic approaches. While identified using a whole-cell high throughput screen (HTS) against replicating bacteria, bedaquiline was found to also have activity against non-replicating *M. tuberculosis* (31, 32). Inspired by the discovery of ATP synthase as the target of bedaquiline, a whole cell phenotypic screen was

subsequently performed to identify additional inhibitors of ATP homeostasis in hypoxic non-replicating *M. tuberculosis* (33). Bryk *et al* used a target-based approach to identify inhibitors of dihydrolipoamide acyltransferase, an enzyme essential for *M. tuberculosis* pathogenesis in guinea pigs, and discovered a group of rhodanine compounds with activity against non-replicating *M. tuberculosis* in a low pH model, a hypoxic model and a combined model incorporating hypoxia, a fatty acid carbon source, mild acidity and nitrosative stress (34). *Mycobacterium smegmatis*, a non-pathogenic and rapid-growing mycobacteria, has been used as a model for *M. tuberculosis* in whole cell screening assays, though this approach is limited by the fact that many active compounds identified in whole-cell screening efforts demonstrate species-specific activity (35). Wang *et al* used a biofilm assay in *M. smegmatis* to identify the small molecule TCA1 (2-acylamino-thiophen-3-carboxamide) with activity against non-replicating *M. tuberculosis* in a biofilm model and in a carbon-starvation model, however the carbon-starvation model used by Wang *et al*, with a three week starvation period entirely in the presence of the small molecule, differs from the model described here (36). Whole-cell phenotypic screening efforts have also been described against non-replicating *M. tuberculosis* utilizing different models of non-replication. Such efforts are technically more difficult with lower throughput relative to replicating whole-cell assays due to the need to establish and maintain the non-replicating state throughout the assay (37). One non-replicating assay based on hypoxia uses a luminescence based low oxygen method and has been used to screen focused chemical libraries (38). A second high-throughput screen against non-replicating bacteria using the combination of reactive nitrogen intermediates, acid, hypoxia and a fatty acid carbon source to induce the non-replicating state, was used to screen a library of ~5,600 compounds, including natural products and known bioactives (39). This screen identified oxyphenbutazone, a nonsteroidal anti-inflammatory drug, as a selective inhibitor of non-replicating *M. tuberculosis*. Starving *M. tuberculosis* of nutrients, which results in a non-replicating, antibiotic tolerant phenotype, has been suggested as a model amenable to HTS (10, 14), however no studies or methods have been published to date using carbon starvation for HTS in *M. tuberculosis*.

In this work, we developed a novel primary assay for HTS with an accompanying secondary assay, using a carbon starvation model for the non-replicating state. The first high-throughput assay, referred to as the primary screening assay, uses GFP fluorescence as a reporter for cell growth and involves a period of exposing starved, non-replicating bacteria to a compound library, followed by a period of outgrowth in rich media. It thus identifies compounds that potentially have activity against replicating and/or non-replicating *M. tuberculosis* as well as compounds that could inhibit the transition from non-replicating to replicating stages of growth. A secondary companion assay, referred to as the luciferase non-replicating assay, uses luciferase expression as a reporter for cell survival and involves exposure of starved, non-replicating bacteria to compounds, without any outgrowth phase. Thus, this assay directly measures the non-replicating activity of compounds. Using the luciferase non-replicating assay together with a replicating assay previously described (35), compounds identified in the primary assay can be characterized as having only replicating activity, only non-replicating activity, or both replicating and non-replicating activity.

We report our experience using these two assays in a pilot screen of 23,680 diverse compounds from the Broad Institute and a subsequent screen of an additional 324,545 compounds at the Southern Research Specialized Biocontainment Screening Center (SRBSBC) as part of the Molecular Libraries Probe Production Centers Network (MLPCN)/NIH program. We also screened two additional sets of compounds, previously identified as having activity against logarithmically growing *M. tuberculosis*, using the luciferase non-replicating assay to determine if any of these compounds additionally had non-replicating activity. Through these screening efforts we identified 786 inhibitors with activity against

non-replicating *M. tuberculosis*. We compared these inhibitors in different *in vitro* models for non-replicating *M. tuberculosis*, specifically the stochastic persister model, the streptomycin dependent model and the low-oxygen recovery assay (LORA). We show that molecules are not uniformly active against all non-replicating models, suggesting that either the ability of molecules to enter cells is different or the essential functions differ in the cell states of the different models. These molecules can thus serve as probes to determine the relevance of the different *in vitro* models to *in vivo* infection.

Results and discussion

Primary screening assay design and results

To identify compounds with activity against nutrient-starved *M. tuberculosis* strain H37Rv, we first optimized starvation conditions for HTS. Betts *et al* had previously described a method for starving the bacteria in phosphate-buffered saline (PBS); however, these conditions result in cell clumping, preventing equal dispersion across a 384-well plate required for HTS (10). We tested several different medias to optimize conditions that yield a non-replicating and antibiotic tolerant phenotype with minimal clumping (Figure S1a), varying the detergent and presence or absence of Middlebrook 7H9. We determined the optimal media to be Middlebrook 7H9 with 0.05% Tyloxapol (7H9/Tx). While this media contains essential cofactors and trace elements, it does not contain sufficient carbon to support growth. Bacterial numbers remain stable over 7 weeks when starved in this media, even when fresh 7H9/Tx is added at 5 weeks mimicking the screening protocol (Figure S1b). We also tested different starvation times and determined that the antibiotic tolerant phenotype is apparent at 2 weeks (Figure S1c). However, previous literature has suggested that the respiration rate of *M. tuberculosis* under starvation conditions continues to decline during the first five weeks of starvation before reaching a plateau (40). We therefore tested outgrowth rates when starved bacteria are returned to rich media and found that bacteria starved for less than 5 weeks have more rapid rates of outgrowth than bacteria starved for 5 weeks or more (Figure S1d). We therefore decided to use 5 weeks as the duration of starvation for the chemical screen.

For the primary screen, we starved *M. tuberculosis* cells containing an episomal, constitutive GFP reporter for five weeks in 7H9/Tx media and then seeded cells at an OD₆₀₀ of 0.05 into 384 well plates containing a compound library (30 μ M). After incubating for 5 days at 37°C, concentrated supplemental media was added to the 384 well plates to restore logarithmic growth conditions. After 4 days of outgrowth, bacterial numbers were assessed using GFP fluorescence as a readout for bacterial growth in the outgrowth phase (Figure 1). While using GFP fluorescence as a readout for bacterial growth may result in the identification of false positives in the case of compounds that inhibit protein expression without inhibiting survival, or false negatives in the case of fluorescent compounds, the use of this readout in conjunction with the luciferase assay described below mitigates these concerns. This screening design, which includes both non-replicating and replicating stages, identifies compounds with activity against both replicating and non-replicating *M. tuberculosis*, non-replicating *M. tuberculosis* alone, replicating *M. tuberculosis* alone, and inhibitors of the transition from non-replicating to replicating stages of growth (Figure 1). The ability of the primary screen to identify compounds with activity against the transition may provide insight into what essential functions are required for the transition from non-replicating to replicating stages of growth, and additionally may suggest a novel therapeutic approach in the treatment of *M. tuberculosis*. Z'-factors for positive control plates range from 0.73–0.92.

We used this primary screening assay in a pilot screen of a library of 23,680 compounds selected from the larger screening collection of the Broad Institute. The complete data from the screen has been deposited in the publicly accessible database ChEMBL (<http://>

chembank.broadinstitute.org/). All compounds were screened in duplicate. Z scores were calculated for each replicate and compared (Figure 2a, Pearson's correlation coefficient = .97). We identified 336 compounds as active (1.4%): 55 bioactive molecules, including many known antimycobacterials (Table S1), 6 purified natural products and 275 commercial compounds (Figure 2b). 6793 compounds were screened in both the primary screen described above and a logarithmic whole-cell screen that we have previously reported (35). Because the primary screen includes 4 days of outgrowth in the same media and growth conditions as the logarithmic screen, we expected that compounds with activity in the logarithmic whole cell screen would also likely exhibit activity in the starvation screen. However, we found that of the 167 compounds exhibiting activity in the logarithmic screen, only 57 compounds were also active in the starvation screen (Figure 2c). This observation suggests that some of the processes required for survival and cellular replication in the first few divisions after non-replicating bacteria resume growth may differ from the processes required for bacteria actively replicating for many generations in the logarithmic phase of growth, or alternatively, that compound entry into cells differs between these two conditions for active replication.

The active compounds identified in the primary screen were picked and retested in 8-point dose response. 271 of the 308 compounds picked (88%) confirmed activity at concentrations at or below the screening concentration (Table S2). We then used a human red blood cell lysis assay to identify compounds with nonspecific toxicity, for example detergents or ionophores. 24 compounds were identified as having nonspecific toxicity and were excluded from further analysis.

Luciferase non-replicating assay design and results

We next wished to further characterize the remaining 247 compounds identified with the primary screening assay to directly determine whether the compounds had non-replicating activity alone, replicating activity alone, non-replicating *and* replicating activity, or activity inhibiting the transition from non-replicating to replicating phases of growth. To directly test for non-replicating activity, we developed a secondary assay, the luciferase non-replicating assay, which does not require outgrowth and therefore only identifies compounds with activity directly against non-replicating bacteria. H37Rv containing a firefly luciferase reporter under the control of the anhydrotetracycline (ATc)-inducible promoter in plasmid pUV15tetORM (41) was starved for 5 weeks in 7H9/Tx and then dispensed into 96 well plates containing compound and ATc to induce luciferase expression. After 5 days the cells were lysed, luciferase reagent added and luminescence measured (Figure S2a). Rifampin and the small molecule gliotoxin, previously identified in a whole cell replicating screen and found to have significant activity against non-replicating *M. tuberculosis*(35), resulted in greater than 95% inhibition of luciferase signal at concentrations of 80× the replicating minimum inhibitory concentration (MIC₉₀) (Figure S2b), while isoniazid (INH) at 80× the MIC₉₀ resulted in only 80% inhibition. We further compared the percent inhibition of luciferase signal to bactericidal activity measured by colony forming units (CFU) for three rifampin and INH concentrations in starved bacilli (Figure S2c,d). For rifampin we found that greater than 95% inhibition in this luciferase assay correlates with almost a 1-log reduction in the number of surviving non-replicating bacteria by CFU, and 99% inhibition correlates with an almost 2-log reduction. Z'-factors for individual control plates ranged from 0.49 to 0.62 for rifampin and from 0.72 to 0.79 for the small molecule gliotoxin. However, Z' factors calculated across multiple control plates were lower, ranging from 0.19 to 0.57, due to plate-to-plate variability in luminescence signal. In order to overcome the plate-to-plate variability in this assay, the % inhibition of luciferase signal for each compound dose was calculated relative to a minimum of 8 DMSO control wells on the same plate. This plate-to-plate variability prevented its use as a primary screen.

When the 247 compounds with confirmed activity in the primary screening assay were tested in 8-point dose response in the luciferase non-replicating assay to determine if they have activity directly on non-replicating bacteria, 162 of the 247 compounds resulted in greater than 95% inhibition. We then tested the 247 compounds in the logarithmic assay previously described (35) and found that 72 compounds display significant activity during logarithmic growth. Combining data from the primary screening assay, the luciferase non-replicating assay, and the logarithmic growth assay allowed us to categorize the compounds according to their spectrum of activities (Figure 3). Of the 247 inhibitors identified, 46 compounds exhibit both replicating and non-replicating activity, 116 exhibit only non-replicating activity, and 26 exhibit only replicating activity. 59 compounds were active in the primary assay (requiring a transition from non-replicating to replicating stages of growth) but do not have activity in either the luciferase starvation assay or the logarithmic growth assay, suggesting that they potentially inhibit the transition. We In addition to secondary assays characterizing their activity spectrum, these 247 active compounds were additionally tested for toxicity against J774 macrophages (Table S2) with 239 compounds demonstrating no significant toxicity at the highest dose tested.

Expanded chemical screening

We expanded screening using the primary screen described above in collaboration with the NIH MLPCN. The screen was performed by the SRSBSC on an additional 324,545 compounds in the Molecular Libraries Small Molecule Repository (MLSMR) library, at a concentration of 25 μ M. Two minor modifications were made to the screening protocol to facilitate BSL3 screening of the large numbers of compounds available in the MLSMR library. First, carbon-starved H37Rv cells were incubated in the presence of compound for only four days prior to adding rich media rather than five, and during the subsequent outgrowth stage the cells were incubated for three days instead of four. Second, resazurin (alarmar blue), a dye that measures metabolic activity as it measures the ability of living cells to reduce resazurin, was used rather than actual measurement of growth (42). 2294 compounds that demonstrated greater than 80% inhibition compared to control wells were defined as active (0.71%). These compounds were picked and 1481(65%) were confirmed to have activity in the primary assay. These 1481 compounds were tested for activity against logarithmically growing bacilli, again using alamar blue as a readout; 894 of 1481 compounds had activity in the secondary logarithmic assay. The 1481 compounds were also evaluated for both chemical promiscuity and the presence of chemically reactive, metabolically labile or hydrolytically unstable functional groups. 750 of the 1481 compounds were selected for further study based on this analysis. When these 750 compounds were retested at the lower concentration of 10 μ M in the original primary carbon starvation assay using GFP fluorescence, 141 compounds were confirmed to have activity (19%). The low retest rate may reflect the lower concentration of the library, protocol differences between the two primary assays, and the use of a different reporter of cell viability (35). 74 of these 141 compounds were repurchased and retested in the primary assay, the luciferase non-replicating assay and the logarithmic growth assay in an 8 point dose response, with a highest concentration of 40 μ M. Of these 74 inhibitors, 55 compounds had activity in at least one of the three assays (74%) (Table S2). These 55 compounds were also tested for cytotoxicity against THP-1 cells. No compounds demonstrated significant toxicity against THP-1 cells at or below 40 μ M (the highest concentration tested) (Table S3).

For the final arm of screening we tested two additional sets of compounds previously identified as having activity against logarithmically growing *M. tuberculosis* in our luciferase non-replicating assay, specifically a collection of 91 compounds identified by Stanley *et al* and a collection of 1113 compounds identified by Ananthan *et al*(35, 43-45). When testing the Stanley set of 91 compounds, previously selected from a logarithmic

screen using glycerol as the primary carbon source and GFP as the reporter, in the luciferase non-replicating assay, we identified 7 compounds with greater than 99% luciferase inhibition (7.7%) (Table S4). In contrast, when we tested the Ananthan set of 1113 compounds (made available by NIAID), selected from a whole cell screen using palmitate as a carbon source and alamar blue as the reporter, we identified 563 compounds with greater than 99% inhibition of luciferase signal (51%) (Table S5). Of the 563 compounds with non-replicating activity, 179 of these had not been identified as having replicating activity when tested in the GFP glycerol logarithmic growth assay (35), despite the fact that the original assay performed by Ananthan *et al* was designed to identify activity against replicating bacteria. Thus, the collection of 1113 compounds appears to be highly enriched for molecules with non-replicating activity. We hypothesize that this difference may be due to the fact that Ananthan *et al* used 7H12 media for their replicating assay. 7H12 contains 5.6 $\mu\text{g/mL}$ palmitate as the primary carbon source and does not result in as robust TB growth as the 7H9/glycerol media used by Stanley *et al* in the GFP logarithmic growth assay (35). This data suggests that replicating screens using fatty acids as a primary carbon source may preferentially select for compounds with activity under both replicating and non-replicating conditions.

Classification of inhibitors of non-replicating, nutrient deprived *M. tuberculosis*

After completing these three arms of screening (the pilot screen, the MLPCN screen, and the rescreening of compounds active in whole cell *M. tuberculosis* replicating assays), we chemically classified the 786 compounds identified with non-replicating activity in the luciferase non-replicating assay. Of these 786, 448 compounds have both replicating and non-replicating activity, and 331 have only non-replicating activity. A selection of the most prevalent chemotypes is shown in Table 1. The largest group of compounds is characterized by a 2-acylamino-1,3,4-oxadiazole core (entry 1, 9% of 786 compounds) followed by another series of 1,3-4-oxadiazole compounds, S-alkyl-1,3,4-oxadiazole-2-thiol analogs (entry 2). Two groups of 20 compounds were identified, aryloxyalkylimidazoles (entry 3) and thiazole-2-amines (entry 4). A family of 7-alkoxy-coumarin or -isocoumarin scaffold was found totaling 19 compounds (entries 5a and 5b). Benzthiazole-2-amine derivatives (entry 6) and benzimidazole-2-amine derivatives (entry 7) represented 1.6% of the 786 compounds. Lastly, smaller groups were characterized by 5-nitrofur-2-carboxamides (entry 8) or S-alkyl-1,2,4-thiadiazoles (entry 9). These 9 major groups represent 25% of the identified non-replicating inhibitors. A search of the literature revealed that 8/9 groups have been previously reported as having replicating activity (all except entry 7) against *M. tuberculosis* in either the Ananthan *et al* HTS or the HTS reported by GlaxoSmithKline against *Mycobacterium bovis* (43, 45, 46) (Table S6). In addition, clusters 1, 5b and 6 were reported by Reynolds *et al* in a screen for inhibitors of replicating *M. tuberculosis* against a chemical library designed around known kinase inhibitors (47). There is no known information about the targets of these inhibitors. Cluster 7 has not previously been reported as having activity against *M. tuberculosis*. Significantly, none of these clusters have previously been characterized or reported as having non-replicating activity.

Comparison in different models of non-replication

In light of evidence supporting the relevance of the non-replicating state *in vivo* during infection (4, 48), numerous *in vitro* models for non-replicating *M. tuberculosis* have been proposed. However, what essential functions can be targeted in the *in vivo* non-replicating state, what the relative importance of the different *in vitro* models are in the context of *in vivo* infection, and whether the physiology of the bacilli across different non-replicating models is identical are all questions that remain unanswered. Toward addressing these questions, we took advantage of the molecules identified in the non-replicating screen and used them to probe the essential functions of the non-replicating bacilli in several different

models. While groups previously have compared the activity of a small set of known antimycobacterials in different models for the non-replicating state, a larger set of unbiased compounds has not been compared in this fashion (13, 37). The prior analysis of antimycobacterials demonstrated that known antibiotics are generally more effective against hypoxic non-replicating cells than against carbon-starved non-replicating cells (37).

We tested 52 molecules identified in the carbon starvation screens in the hypoxic low oxygen recovery assay (LORA) (38). Of the 52 molecules tested with a luciferase MIC₉₀ less than 40 μM in the carbon starvation assay, only 9 had MIC₉₀s less than 40 μM in the LORA assay, and only 17 had MIC₉₀s less than 100 μM (Figure 4 and Table S3). 96% of the small molecules identified using the luciferase non-replicating assay were more active in the carbon starvation assay than in the LORA assay, and only 33% of the small molecules were active in both the LORA and carbon starvation assays. Taken together, these data demonstrate that the *in vitro* model used to identify inhibitors of the non-replicating state greatly affects the inhibitors that are identified. We hypothesize that lack of correlation between models suggests physiologic differences between the cells, either changes in the cell envelope that affect small molecule permeability, or differences in cellular functions essential for survival under these different conditions. While some inhibitors of the non-replicating state may be active across models, for example the rhodamine inhibitors of dihydrolipoamide acyltransferase identified by Byrk *et al*, many non-replicating inhibitors may be specific for a single model (34).

We next selected 4 molecules highly active under both carbon starvation and low oxygen conditions for further study (Figure 5): 3-(*m*-tolyl)-5-((1-piperidinyl)carbonylmethyl)thio-1,2,4-thiadiazole (**1**, from group 9), *N*-(4-chlorophenyl)-5-(1-methyl-5-(trifluoromethyl)-1*H*-pyrazol-3-yl)thiophene-2-carboxamide (**2**, singleton), 2-((2-(1,3-dioxan-2-yl)ethyl)thio)-5-phenyl-1,3,4-oxadiazole (**3**, from group 2) and *N*-(3-methylpyridin-2-yl)-4-(pyridin-2-yl)thiazol-2-amine (**4**, from group 4). These molecules represent three major structural families assigned by the chemical classification of inhibitors as well as one potent unique structure. Only compound **4** has been reported previously as having activity against replicating *M. tuberculosis*, and none have been reported as having non-replicating activity. We measured the minimum inhibitory concentration (MIC₉₀) for each compound against replicating *M. tuberculosis* grown in Middlebrook 7H9 media (Figure 6a) using OD₆₀₀ as a measure of growth. We then measured the minimum bactericidal concentration (MBC₉₀) for each compound against non-replicating carbon-starved *M. tuberculosis* (Figure 6a and S3a-d), enumerating surviving bacteria by colony forming units (CFU). We found that for these four molecules, the MBC₉₀s against non-replicating bacteria are equivalent or better than the MIC₉₀s against actively replicating bacteria (Figure 6a). In addition, compounds **1** and **4** display sterilizing activity against non-replicating bacteria (Figure S3a-d). The fact that these molecules demonstrate equivalent or increased potency against non-replicating bacteria compared to replicating bacteria is in contrast to all antibiotics currently used to treat tuberculosis. To date all antibiotics active against *M. tuberculosis*, including rifampin and bedaquiline, demonstrate at least a 10-fold decrease in potency against carbon-starved non-replicating bacteria compared to replicating bacteria (13, 14). In addition, no antibiotics currently being used to treat tuberculosis exhibit such low MBC₉₀s against carbon-starved non-replicating bacteria.

We then tested Compounds **1-4** in two additional models for the non-replicating state, the stochastic persister model and the streptomycin dependent model. While we recognize that persisters may not represent a non-replicating population, these cells are antibiotic tolerant and thus represent another population which may be important during *in vivo* infection. Using a system we have previously established, we tested compounds **1-4** in the stochastic persister model (16). Only one of the four compounds, thiadiazole **1** exhibits significant

activity against the antibiotic tolerant persister population at a high dose ($5\times$ the starvation MBC_{90} , p value = .0003) (Figure 6b), but not at the starvation MBC_{90} (Figure S3b). When the molecules were tested in the streptomycin dependent model, two compounds, **1** and aminothiazole **4**, exhibit significant activity against the non-replicating population at $5\times$ the starvation MBC_{90} (Figure 6c) while **2** and **3** did not. When tested at the starvation MBC_{90} , **1** has no activity against the streptomycin-deprived cells, while **4** still exhibits significant activity (Figure S4). The widely varying activities of these molecules within these different models for the non-replicating state suggest that these models represent different bacterial physiologic states. It is unclear which model is most useful for predicting *in vivo* activity; however, knowing if one model is better than others would be enormously valuable to tuberculosis drug discovery efforts. We suggest that compounds identified in this work provide chemical tools that can be used to answer this question. In addition, some molecules displaying activity in multiple models, given the current limitations of our understanding, may represent molecules that should be prioritized for further *in vivo* study.

Conclusions

In this report we describe a novel HTS assay using nutrient, specifically carbon, starvation as a model for the non-replicating state in *M. tuberculosis* and a secondary assay that directly measures activity against non-replicating bacteria. The primary screening assay can be used to identify compounds with activity against replicating and non-replicating *M. tuberculosis* as well as compounds that inhibit the transition from non-replicating to replicating stages of growth. The follow-up luciferase non-replicating assay directly measures activity against *M. tuberculosis* in the absence of any carbon-source. We report our experience using these assays to screen $\sim 24,000$ diverse compounds from the Broad Institute as well as a subsequent screen of an additional 324,545 compounds as part of the MLPCN/NIH program. In addition to providing potentially promising scaffolds and prioritization of previous screening efforts, several important lessons emerged.

In testing 6793 compounds from the Broad Institute compound collection in both the primary screen described in this work, and a logarithmic whole cell screen previously described (35), we found that only 37% of compounds with activity in the logarithmic screen were also active in the primary screen, despite the presence of an outgrowth stage in the primary assay using the same media and growth conditions. This observation suggests that bacilli during the first few divisions transitioning from non-replicating to replicating stages of growth differ from bacilli in mid-logarithmic growth phase with respect to small molecule sensitivity. These differences may result because they have differing essential functions, or alternatively, differences in cell wall permeability. There is increasing evidence to suggest that under non-replicating conditions the mycobacterial cell envelope undergoes remodeling and may become less permeable to small molecules (49). Thus it is possible that the cell envelope during the first few divisions out of the non-replicating state may be less permeable to these small molecules that have activity during mid-logarithmic growth phase. The small molecules identified in this work could be used to test this hypothesis using the metabolomics approach recently described by Chakraborty *et al* to determine intracellular concentrations for different antibiotics in *M. tuberculosis*(50).

In this work we also tested two collections of compounds previously identified in different replicating whole cell screens in the luciferase carbon-starvation assay. We found that these different logarithmic assays yielded very different numbers of compounds with non-replicating activity, ranging from 9% from a glycerol-GFP logarithmic assay to 51% in a palmitate-alarmar blue logarithmic assay. From this observation we hypothesize that using different growth conditions and perhaps different reporters in a replicating screen may strongly bias the number of replicating inhibitors identified that also demonstrate non-

replicating activity. Currently it is unclear which of these *in vitro* logarithmic screening conditions is more relevant for *in vivo* infection, but testing the identified chemical probes in an *in vivo* infection model would shed light on this issue.

Using the two assays described in this work we identified 786 molecules with activity against *M. tuberculosis* in which non-replication was induced by nutrient starvation. Among these molecules, we identified 9 significant groups in which 25% of identified inhibitors could be chemically categorized. We tested 52 molecules with activity in the luciferase non-replicating assay in the hypoxia-based LORA assay, and found that only 33% are active in both assays. We selected 4 molecules highly active in both the luciferase non-replicating assay and LORA and found that these molecules exhibit equal or increased potency against non-replicating bacteria compared to replicating bacteria. In addition, the carbon starvation MBC_{90} s for these molecules (2.4 μM to 6.5 μM) are within the range of values reported for RIF (range between .8 μM and 12 μM) and significantly lower than the carbon starvation MBC_{90} s reported previously for other TB drugs, including bedaquiline (90 μM) and moxifloxacin (>100 μM) (13, 14, 37).

Important questions underlying the pathophysiology of *M. tuberculosis* infection include what role the non-replicating state plays *in vivo* during TB infection, and whether the non-replicating state contributes to the long treatment duration required for cure. Answers to these questions have the potential to guide more effective drug discovery efforts. However, it is currently unclear which *in vitro* model is most relevant for *in vivo* infection, and how many essential functions required for the survival non-replicating bacteria are shared between the different models. While we know from studies with the Rel_{MTB} mutant that the starvation stress response is important for chronic infection in mice and guinea pigs (23-25), it is interesting to note that most current antituberculars are ineffective against nutrient-starved bacilli, while many of them have some activity against hypoxic bacilli (13, 37). Furthermore, three of the most promising new treatments for *M. tuberculosis* infection, the nitroimidazole PA-824, bedaquiline, and the recently re-discovered antibiotic clofazimine, which animal data suggests may shorten the required duration of therapy (28, 30), demonstrate activity against not only hypoxic, but also nutrient-starved *M. tuberculosis* (14, 37). These observations raise the interesting possibility that these are promising candidates because of their activity against nutrient-starved, rather than hypoxic bacilli or because they have activity against both nutrient-starved and hypoxic bacilli. Given that relatively few small molecules currently target nutrient-starved bacilli, identifying inhibitors that are specific to nutrient-starved *M. tuberculosis* will allow testing of this hypothesis.

In this work, we found that there may be potentially limited overlap between compounds with activity against carbon-starved bacilli and additional models for the non-replicating state. Therefore, easier surrogate, non-replicating models will not substitute for assays designed to specifically target carbon-starved *M. tuberculosis* bacilli. Whether these differences in activity between models represent different cellular permeabilities or different essential functions is unclear. Nonetheless, they illustrate physiological differences among the cells in these different models. The chemical tools described in this work will be invaluable to further probe the relevance of the different non-replicating models by testing their *in vivo* efficacy in murine models of infection and may help to identify and validate essential *in vivo* functions and targets. In the meantime, we have identified several inhibitors with activity in multiple models of the non-replicating state that are more active against non-replicating, nutrient starved *M. tuberculosis* than current tubercular drugs. Based on our current understanding of TB infection, these molecules may represent more promising candidates for further investigation and therapeutic development.

Materials and Methods

Bacterial strains and growth conditions

The strain *M. tuberculosis* H37Rv was used for all experiments. GFP was expressed using a constitutive episomal plasmid (hygromycin selection) driven by the Rv3583c promoter. An inducible firefly luciferase expression plasmid (kanamycin selection) was constructed using an ATc inducible system, as described previously (41). *M. tuberculosis* was grown at 37°C in Middlebrook 7H9 broth supplemented with 10% OADC (oleic acid-albumin-dextrose complex), 0.2% glycerol and .05% Tween-80 or on Middlebrook 7H10 plates supplemented with 10% OADC enrichment. The INH MIC₉₀ against replicating H37Rv was 0.5 μM and the RIF MIC₉₀ was .01 μM.

Carbon Starvation

Freezer stocks of H37Rv were diluted 1:50 in fresh 7H9 OADC media containing the appropriate selective antibiotic and cultured until OD₆₀₀ between 0.6 and 1.0. The bacteria were centrifuged at 2800 × g for 5 minutes and resuspended in 50 mL of starvation media (7H9 powder and .05% Tyloxapol without any supplementation or antibiotic selection). The cells were then washed an additional two times with 7H9/Tx, resuspended to an OD₆₀₀ of 0.2 and a 50 mL culture was aliquoted into a sterile roller bottle. The starvation culture was incubated standing at 37°C for 5 weeks.

Primary screening assays

For *M. tuberculosis* starvation screening assays, starved bacteria expressing GFP were diluted and plated into 384 well plates containing compound for a final OD₆₀₀ of 0.05, a final volume of 40 μL and a final compound concentration of 30 μM. Plates were incubated for 120h at 37°C, at which time 10 μL 5× concentrated media was added to each well (7H9 media with 50% OADC, 1% glycerol, .05 % tyloxapol) without mixing. Plates were then incubated for an additional 96 hours, at which time fluorescence was read using an M5 Spectramax (excitation λ = 470nm, Emission λ = 509nm). Each compound was screened in duplicate, and composite z-scores were calculated using DMSO controls as reference. Hits from the *M. tuberculosis* screen were defined as compounds with a composite z-score of less than -6. This z-score was the z-score for the concentration of the control antibiotic rifampicin that gave a Z'-factor of 0. When the starvation screening assay was performed at SRI several modifications were made. Plates containing *M. tuberculosis* and compounds were incubated for 96 hours prior to the addition of concentrated media. Plates were then incubated for an additional 72 hours, at which point Alamar Blue was added to the plates. The plates were then incubated overnight at 37°C prior to reading with an Envision plate reader (excitation λ = 531, emission λ = 509nm). Hits for the SRSBSC screen were defined as compounds that resulted in 80% inhibition compared to control wells.

Compound Library

The 24,000 compounds screened against *M. tuberculosis* were obtained from the Broad Institute small molecule library. The categories of compounds were as follows: 40% commercially available compounds purchased predominantly from ChemDiv (ChemDiv, San Diego, CA), Maybridge (Thermo Fisher Scientific, Cornwall, UK) and TimTec (TimTec, Newark, DE); 30% compounds synthesized by Broad chemists using Diversity Oriented Synthesis (DOS) or other strategies for organic synthesis; 30% natural products consisting of natural product extracts and purified natural products.

Luciferase non-replicating screen

Hits from the primary screen against *M. tuberculosis* were cherry picked from the library and arrayed into 96 well plates as a dose response curve using 2 fold serial dilutions from the primary screening concentration. The edge wells were left empty given edge effects in luminescence. ATc (50 ng/mL) was added to the carbon-starved H37Rv culture containing the inducible firefly luciferase plasmid and 80 μ L dispersed into assay plates. After 5 days the cells were lysed with the addition of 20 μ L of Cell Culture Lysis 5 $\times$ Reagent (Promega Corporation) and 5 mixing cycles (full volume aspirate and dispense). After 10 minutes 100 μ L of luciferase was reagent added and luminescence immediately measured (Luciferase Assay System, Promega Corporation). Rifampin at 80 $\times$ the MIC was used as a positive control for the assay. The average luciferase signal for DMSO control wells (minimum of 8 wells) was calculated for each plate, and the % inhibition of luciferase signal was calculated for each compound relative to the average DMSO signal on the same plate. Z'-factors for individual control plates ranged from 0.49 to 0.62.

MIC₉₀ and MBC₉₀ determinations

To determine replicating MIC₉₀ values, bacteria were grown to mid-log phase and plated in 96 well plates at OD₆₀₀ = 0.025 in the presence of specified concentrations of small molecule inhibitors for indicated time periods, and growth was assessed by reading OD₆₀₀. The MIC₉₀ was defined as the minimum concentration that inhibited growth by 90% relative to the DMSO control. To determine starvation MBC₉₀s, carbon-starved bacteria were diluted to OD₆₀₀ = .05 in starvation media and incubated in the presence of small molecule inhibitors for indicated time periods. Surviving bacteria were enumerated by plating for CFUs.

Testing in different *in vitro* non-replicating models

Testing compounds in the persister model was done as previously described (16). Briefly, an H37Rv culture in mid log phase was diluted to an OD₆₀₀ of 0.2 and 12 mL of the diluted culture was then aliquoted in square media bottles. Ofloxacin at 1 μ g/mL and INH at 0.1 μ g/mL were added. Three duplicate media bottles were set up for each time point measured. At indicated time points 300 μ L of cells were removed from each media bottle being assayed. Collected cells were washed once in fresh media, serially diluted and plated on 7H10 plates. For challenge with small molecules, square media bottles were covered with rubber septa (Sigma) and compounds were injected through the septum at the indicated concentration in 0.5 mL of media. Rifampin, tested at 0.1 μ M (10 $\times$ the replicating MIC₉₀), is shown as a control. For testing in the streptomycin dependent model, the 18b strain was grown as previously described (21, 22). The streptomycin starved cells were diluted to OD₆₀₀ = .05 and incubated in 96 well plates in the presence of a 2-fold dose response curve of compounds for 7 days. Surviving bacteria at the indicated concentrations were enumerated by plating for CFUs on 7H10 agar containing streptomycin. The activity of INH, tested at 0.5 μ M (1 $\times$ MIC₉₀) and 5 μ M (10 $\times$ MIC₉₀), is shown as a control. The LORA assay was performed as previously described(38).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J. Bittker, C. Mulrooney and the Broad Institute Chemical Biology platform for technical assistance and data management; R. Goldman, B. Laughon, T. Parker, the NIAID and TAACF/SRI for providing the 1113 compound set; Lucille White and SRSBSC for completing the primary starvation screen and secondary logarithmic screen against the 324,545 MLSMR library; J. Gomez for helpful discussion and advice throughout the project; A.

Campos-Neto for the *M. tuberculosis* 18b strain. SSG gratefully acknowledges the NIH for funding K08AI085033. This work was supported through funding from the Bill and Melinda Gates Foundation to DTH, NIH R03MH087444 to DTH, and by the NIH Molecular Libraries Production Centers Network (MLPCN; www.mli.nih.gov), which funded primary screening of a 324,545-compound library against *M. tuberculosis* strain H37Rv at SRI.

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Figure 1. Schematic of the high-throughput primary GFP screening assay using carbon-starved *M. tuberculosis*

Because the assay contains an outgrowth step, inhibitors identified with this assay may exhibit non-replicating activity only, both non-replicating and replicating activity, replicating activity only, or finally may inhibit the transition from the non-replicating stage to the replicating stage of growth.

Figure 2. Results from the pilot screen

A. All screening was performed in duplicate, and the correlation between calculated z scores for each duplicate is shown. **B.** Using a z-score cutoff of -6, 336 compounds were identified as hits (1.4%). The distribution of z scores for the pilot screen is shown. **C.** 6793 compounds were screened in both the primary carbon starvation assay (which includes both non-replicating and replicating stages) and the logarithmic replicating growth assay. The correlation of hits between the two assays is shown. Of the 167 hits identified using the logarithmic replicating growth assay, only 57 were also hits in the primary carbon starvation assay (shaded quadrant).

Figure 3. Flow-chart demonstrating the secondary assays used to characterize active compounds

Of the 247 hits, 46 compounds were identified as having both replicating and non-replicating activity, 116 as having only non-replicating activity, and 26 as having only replicating activity. In addition, 59 compounds retested in the primary assay but did not exhibit activity in the replicating or non-replicating secondary assays, suggesting these compounds may inhibit the transition from the non-replicating to replicating state.

Figure 4. Testing inhibitors in the hypoxic LORA assay

52 molecules MIC₉₀s less than 40 μM in the luciferase carbon-starvation assay were tested in the hypoxic LORA assay. Only 9 of the 52 molecules also had LORA MIC₉₀s less than 40 μM .

Figure 5. Structures of four novel molecules with replicating and non-replicating activity
1 is a member of group #9 (Table 1), **2** is a singleton, **3** from group #2 and **4** is from group #4.

Figure 6. Further characterization of four highly active inhibitors

A. The LORA MIC₉₀ (μM) starvation MBC₉₀ (μM) and replicating MIC₉₀ (μM) for compounds **1-4**. The ratio of replicating to non-replicating activity reflects the fact that compounds **1-4** are more potent against carbon-starved bacteria than replicating bacteria. **B,C.** Compounds **1-4** were tested at a concentration of 60 μM in the stochastic persister model (**B**) and the streptomycin dependent model (**C**). Bars represent the average of three biologic replicates and error bars represent the standard deviations.

Table 1
Clustering of molecules with non-replicating activity in the carbon-starvation luciferase non-replicating assay

The 786 molecules with non-replicating activity were clustered. The 9 largest clusters are shown. The activity profile reflects the representative molecule shown.

#	Substructure type	Representative structure	# members	Activity profile
1	$R_1, R_2 = \text{alkyl, cycloalkyl, aryl, heteroaryl}$		70	Nonreplicating
2	$R_1, R_2 = \text{alkyl, cycloalkyl, aryl, heteroaryl}$		30	Replication and nonreplicating
3	$R_1 = \text{alkyl, aryl, heteroaryl, alkyl}; R_2 = \text{alkyl, aryl, heteroaryl, n = 0-3}$		22	Replication and nonreplicating
4	$R_1 = \text{aryl, heteroaryl, alkyl}; R_2 = \text{aryl, heteroaryl}$		21	Replication and nonreplicating
5a	$R_1 = \text{H, alkyl, aryl}; R_2 = \text{H, alkyl, aryl, alkoxy}; R_3, R_4 = \text{alkyl}$		11	Replication and nonreplicating
5b	$R_1 = \text{H, alkyl, aryl}; R_2 = \text{H, alkyl, aryl, alkoxy}; R_3, R_4 = \text{alkyl, Cl}$		8	Replication and nonreplicating
6	$R_1 = \text{Cl, F, OMe, Me}; R_2 = \text{alkyl, aryl}$		11	Nonreplicating
7	$R_1 = \text{alkyl, acyl}; R_2 = \text{H, alkyl}$		10	Replicating and nonreplicating
8	$R_1 = \text{aryl}$		7	Replicating and nonreplicating
9	$R_1 = \text{alkyl}; R_2 = \text{aryl}$		7	Replicating and nonreplicating