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Targeting phenotypically tolerant *Mycobacterium tuberculosis*

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Abstract

While the immune system is credited with averting tuberculosis in billions of individuals exposed to *Mycobacterium tuberculosis*, the immune system is also culpable for tempering the ability of antibiotics to deliver swift and durable cure of disease. In individuals afflicted with tuberculosis, host immunity produces diverse microenvironmental niches that support suboptimal growth, or complete growth arrest, of *M. tuberculosis*. The physiological state of nonreplication in bacteria is associated with phenotypic drug tolerance. Many of these host microenvironments, when modeled *in vitro* by carbon starvation, complete nutrient starvation, stationary phase, acidic pH, reactive nitrogen intermediates, hypoxia, biofilms, and withholding streptomycin from the streptomycin-addicted strain SS18b, render *M. tuberculosis* profoundly tolerant to many of the antibiotics that are given to tuberculosis patients in a clinical setting. Targeting nonreplicating persisters is anticipated to reduce the duration of antibiotic treatment and rate of post-treatment relapse. Some promising drugs to treat tuberculosis, such as rifampicin and bedaquiline, only kill nonreplicating *M. tuberculosis* in vitro at concentrations far greater than their minimal inhibitory concentrations against replicating bacilli. There is an urgent demand to identify which of the currently used antibiotics, and which of the molecules in academic and corporate screening collections, have potent bactericidal action on nonreplicating *M. tuberculosis*. With this goal, we review methods of high throughput screening to target nonreplicating *M. tuberculosis* and methods to progress candidate molecules. A classification based on structures and putative targets of molecules that have been reported to kill nonreplicating *M. tuberculosis* revealed a rich diversity in pharmacophores. However, few of these compounds were tested under conditions that would exclude the impact of adsorbed compound acting during the recovery phase of the assay, and few were tested under more than one condition imposing nonreplication. That nonreplicating mycobacteria are metabolically active was corroborated by their susceptibility to several antibiotics and tool compounds that target the synthesis of lipids, RNA, DNA, proteins, and peptidoglycan.

1. Introduction

Two parallel revolutions were born in the golden era of antibiotics (~1940–1960). One was a revolution in medicine, as physicians went to war with microbes. The second was a revolution in biology, as microbiologists and geneticists used anti-infectives as tools to reveal how microbes functioned on a molecular level. Scientists converged on a surprisingly

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short list of essential biological processes that appeared to make up an Achilles' heel shared by diverse bacterial pathogens— the biosynthesis of nucleic acids (DNA and RNA), protein, cell walls (peptidoglycan and lipids), and folate. Only later were the far wider dimensions of potential target space appreciated (1). The discovery of targets led to the development of methods to improve existing antibiotics and find new ones.

The success of chemical biology at advancing antibiotic development was spectacular but short-lived. New antibiotics quickly encountered genetically-encoded drug resistance (2). The selective pressure imposed by antibiotics presented bacteria with a seemingly impossible task of becoming drug resistant by modifying the antibiotic's target, modifying the antibiotic's structure, effluxing the antibiotic, or altering their cell wall's permeability to the drug without a major fitness cost. Yet bacteria solve this problem routinely in laboratories, the environment, animal models of disease, and patients. Resistant mutants distribute drug resistance by vertical transmission (passing chromosomal DNA to their progeny) and horizontal transmission (via phages and plasmids). Antibiotic research concentrated on understanding the basis of genetically encoded drug resistance and medicinal chemistry campaigns focused on bypassing it.

However, genetic drug resistance was not the only hurdle. Pioneering observations published by Hobby, Meyer and Chaffee in 1942 (3), and Bigger in 1944 (4) cast an ominous cloud over the remnants of optimism that antibiotics could eradicate diseases of bacterial origin. Hobby and her colleagues observed that about 1 streptococcus of 10^6 in a replicating culture survived exposure to penicillin, while in a culture whose replication was halted by cold, nearly all the cocci survived (3). Bigger made the same observation with staphylococcus and additionally noted that the cocci became tolerant to penicillin when their replication was halted by acidification or hypotonicity of the medium (4). Microbiologists had long assumed that logarithmically growing bacterial cultures were uniform. Use of penicillin as a tool allowed Hobby, Meyer, Chafee and Bigger to discover that the assumption of bacterial homogeneity was incorrect. Moreover, they demonstrated that bacteria could resist killing by antibiotics through a non-heritable mechanism. The penicillin-resistant cells were as sensitive to penicillin as the population from which they came when they were expanded in fresh medium and exposed to penicillin a second time. Bigger used the term "persisters" for bacteria that survived antibiotics without heritable resistance. The property allowing persisters to survive was later termed "phenotypic drug resistance", or, "phenotypic tolerance". These historic studies have important implications for anti-infective discovery paradigms today (3, 4).

Two decades later, Hobby and Lenerts extended the observation of phenotypic tolerance to a different organism, *Mycobacterium tuberculosis*, and additional drugs, isoniazid and para-aminosalicylate (5). Isoniazid targets the synthesis of mycolic acids, para-aminosalicylate targets the synthesis of folate and penicillin targets the synthesis of peptidoglycan. Thus, the phenomenon of phenotypic tolerance was independent of the chemical class of antibiotics and of the pathways they inhibit.

The problem of persisters is central to the chemotherapy of tuberculosis. It is believed to be a major reason why the current WHO-approved treatment regimen for drug-sensitive

tuberculosis takes 6 months to achieve cure in ~95% of participants in formal studies; the cure rate is about 86% in routine practice. Drug-resistant tuberculosis generally requires treatment for over two years and cure is often not achieved (6). In the “Cornell model”, mice with drug-sensitive tuberculosis that are treated with isoniazid and pyrazinamide for two months harbor no detectable colony forming units of *M. tuberculosis* when their organ homogenates are spread on bacteriologic agar. However, about one-third of the remaining members of the same cohort of mice relapse spontaneously some months later, and nearly all of them relapse if immunosuppressed with corticosteroids, anti-IFN γ , anti-TNF, or inhibitors of inducible nitric oxide synthase (7–9). The *M. tuberculosis* recovered at relapse is as sensitive to isoniazid and pyrazinamide as the population used for inoculation. These observations indicate the presence of drug tolerant persister populations after antibiotic treatment, even if they are temporarily undetectable by standard microbiologic methods. Likewise, sputa from about 80% of treatment-naïve tuberculosis patients contained *M. tuberculosis* that were not quantifiable by CFU analysis (10, 11)

Experience with metronidazole illustrates the challenge of translating the foregoing knowledge into a faster and more effective treatment of tuberculosis. In some animal models, *M. tuberculosis* encounters hypoxia in necrotic granulomas (Table 1). *In vitro*, hypoxia causes mycobacteria to cease replicating and become phenotypically tolerant to most drugs. In contrast, the anti-bacterial and anti-parasitic drug metronidazole kills hypoxic mycobacteria *in vitro*. Thus metronidazole seemed well suited to kill nonreplicating *M. tuberculosis*. However, metronidazole’s activity in animal models of tuberculosis correlated imperfectly with hypoxia in granulomas (Table 1) (12–19). Metronidazole improved the proportion of patients whose sputum became smear- or culture-negative at one month of treatment, but did not impact treatment outcome at 6 months, other than contributing to peripheral neuropathy (14). In retrospect, the ability of metronidazole to kill hypoxic *M. tuberculosis* *in vitro* was studied in the absence of an alternate electron acceptor, putting the organism at a greater disadvantage than it is likely to face *in vivo*. *M. tuberculosis* is not restricted to using oxygen as an electron acceptor; it can also use nitrate or fumarate (20–22). Nitrate is a physiologic constituent of human body fluid. Inclusion of nitrate markedly diminished the *in vitro* efficacy of pyrazinamide (23).

The experience with metronidazole suggests that it may not be enough to find antibiotics with the exceptional property of killing bacteria that are phenotypically tolerant to most other antibiotics; it matters how the bacteria are rendered phenotypically tolerant. If phenotypic tolerance is achieved by using conditions that prevent the bacteria from replicating, it matters how they are prevented from replicating. The more the conditions resemble those in the host, the more likely that drugs that work under those conditions may also work in the host.

The foregoing statements are a hypothesis whose testing is just beginning. After Bigger’s report (4), it took another 40 years until Coates proposed large-scale screening to target nonreplicating *M. tuberculosis* (24). His proposal came at time when many pharmaceutical companies were scaling back or abandoning anti-infective discovery. Other firms stuck to the industry’s standard practice of seeking broad-spectrum agents that could cure infections prevalent in economically advantaged regions. Only after 1999 did a new funding landscape

emerge that supported academic–industrial partnerships for drug discovery for infectious diseases that are prevalent chiefly in economically disadvantaged regions (1, 25–27). Only about 10 years ago did pharmaceutical companies and their academic partners begin large-scale screens for drugs targeting phenotypically tolerant mycobacteria (28, 29).

This chapter describes and categorizes approximately one hundred compounds that have been reported to kill mycobacteria rendered nonreplicating in one or another *in vitro* model. We also offer comments about the biology of drug tolerance, strategies for screening compounds against phenotypically tolerant mycobacteria and progressing the actives through secondary assays, and pitfalls in data interpretation.

2. Summary of key observations

- At least two types of phenotypic tolerance are important to distinguish because different strategies will probably be needed to overcome them (25, 27). Class I phenotypic tolerance is manifest by a small proportion of bacteria in a replicating population. Existing evidence, while incomplete, suggests that different individual bacteria in the population can be phenotypically tolerant to different antibiotics by different mechanisms. To the degree that this is the case, phenotypic tolerance can be overcome by treating the overall population with a combination of different drugs. Class II phenotypic tolerance is manifest by almost all the cells in a population whose number is not changing during the period of observation. Almost every cell must be phenotypically tolerant to all the antibiotics to which the population as a whole is tolerant. Overcoming class II phenotypic tolerance will likely require new kinds of drugs that are highly active on nonreplicating bacteria
- While nonreplication imposed by diverse stresses is closely associated with phenotypic tolerance, mycobacteria that become nonreplicating under different conditions may be phenotypically diverse.
- While nonreplication is a state associated with phenotypic tolerance, nonreplication is not a mechanistic explanation for phenotypic tolerance. Nonreplication is not equivalent to dormancy and does not connote a lack of dependence on biosynthetic pathways.
- Nonreplicating mycobacteria are reportedly killed by a large number of chemically diverse compounds. However, only a few of these compounds have been tested and found active against mycobacteria rendered nonreplicating in multiple ways.
- Only a few of the compounds reported to kill nonreplicating mycobacteria have been tested under conditions designed to exclude false-positive results that can arise from drug carry-over from the nonreplicating phase of the assay to the replicating phase, such as by enumerating viable bacilli on solid bacteriologic medium containing activated charcoal (30–33).

- Some approved drugs for tuberculosis, such as rifampicin and moxifloxacin, are genuinely active *in vitro* against nonreplicating *M. tuberculosis*, but at far higher concentrations and with far less reduction in bacterial numbers than under replicating conditions. For others, such as bedaquiline, the apparent activity against nonreplicating *M. tuberculosis in vitro* was largely attributable to carry-over in one study (30). Among the approved tuberculosis drugs tested in that study, only PA-824 was genuinely and comparably active against *M. tuberculosis* under replicating and nonreplicating conditions (30). Encouragingly, ongoing research is identifying more such compounds among anti-infectives approved for other indications (34) or as new members of drug-like chemical classes.

3. Diversity in nonreplication

Sensitivity to an antibiotic is conventionally defined under replicating conditions and reported as a minimum inhibitory concentration (MIC), typically meaning a concentration that restricts growth by at least 90% compared to a culture under the same conditions that is exposed to the vehicle alone for the same period of time. Phenotypically tolerant bacteria of class I are those rare cells that survive exposure to the antibiotic at or above its MIC when tested under these standard, replicating conditions. In contrast, phenotypically tolerant bacteria of class II are the majority of a population that survives exposure to the antibiotic at or above its MIC under different conditions, typically those that impose nonreplication. To distinguish the nonreplication imposed by the test conditions from death imposed by the antibiotic usually requires removing the antibiotic by washing or dilution and reversing the conditions that impose nonreplication, then detecting recovery or the lack of recovery of the surviving bacteria by allowing survivors to replicate. The hallmark of both class I and class II phenotypic tolerance is that the survivors, when tested again under the standard conditions, display the same MIC as the original population (25).

To fully appreciate the diversity exhibited by nonreplicating cells, it will be useful to start by correcting several misconceptions. First, when Hobby et al. (3) and Bigger (4) discovered what we now call class I persistence, they attributed it to nonreplication of about 1 bacterium in a million in an otherwise replicating population. They had no direct evidence for this. More than half a century later, it became possible to test this notion, and the results have been mixed. In short, class I phenotypic tolerance sometimes is and sometimes is not associated with nonreplication of a minority of cells in a replicating population. By definition, class II persisters are nonreplicating. Therefore, class I and class II persisters should not be grouped together as “nonreplicating cells”. Second, just because a bacterial population has stopped changing in number over a period of time does not exclude the occurrence of balanced replication and death. For simplicity, we use the term “nonreplication” to describe a population of static size, but without implication as to the degree of turnover. Third, just because one population of bacteria has entered a nonreplicating state in response to one condition does not mean that it has the same phenotype as another population that has entered a nonreplicating state in response to another condition.

Single-cell analyses of persister populations are now feasible. For example, cell division can be monitored by dilution of a fluorescent signal from a chromosomal copy of mCherry (35, 36), and metabolism monitored using redox sensor green (RSG), which generates a fluorescent signal upon reduction by bacterial reductases. The fates of individual cells can be tracked over time by microfluidics and time-lapse microscopy (36–39). Replicating and nonreplicating cells, and metabolically active and metabolically inactive cells, may be sorted using FACS. For example, Brynildsen and colleagues found that while non-growing cells were enriched for class I persisters, 20% of the persisters were replicating, and slow metabolism correlated with, but was not required, for persistence (36).

Persister diversity may result from heterogeneity in such bacterial processes as maintenance of membrane potential, DNA replication, and ribosomal translation (40, 41), or in host environments, where bacteria may be extracellular in connective tissue or caseum, or intracellular in phagosomes or cytosol (42). The transcriptome of *M. tuberculosis* class I persisters enriched by D-cycloserine treatment to kill replicating cells overlapped very little with the transcriptomes of class II phenotypically tolerant cells generated by incubating *M. tuberculosis* under conditions of hypoxia (43, 44), stationary phase (12, 44, 45), or nutrient starvation (12). Moreover, only 5 genes were identified as commonly upregulated in the 4 nonreplicating models (46) and there was little overlap of *M. tuberculosis*'s differentially regulated genes in the 3 class II nonreplicating models (43, 44, 46). Another comprehensive comparison found a poor correlation of transcriptomes of *M. tuberculosis* rendered nonreplicating in multiple models, including removing streptomycin from the streptomycin-addicted strain SS18b, exposing wild type *M. tuberculosis* to reactive nitrogen intermediates, depriving it of phosphate, nutrients or oxygen, or combining a variety of stresses (47). On the other hand, Voskuil et al. found a correlation among the transcriptomes of *M. tuberculosis* exposed to hypoxia, the nitric oxide donor DETA-NO, and cyanide (48), and the transcriptional changes were similar to those seen during *M. tuberculosis*'s infection of IFN γ -activated bone-marrow derived macrophages (49). While these transcriptomics experiments were insightful, we do not know the relevance of transcriptional regulation of individual genes to the survival of mycobacteria as class I or class II phenotypically tolerant. Transcriptomics profiles are time-dependent, making it difficult to compare transcriptomes studied at different times. Moreover, many key regulatory steps are post-translational.

Another indication of the diversity of nonreplicating mycobacteria is that the same compounds are differentially active against *M. tuberculosis* rendered nonreplicating in different ways, such as nutrient starvation, stationary phase, hypoxia, and a combination of acidic pH and reactive nitrogen intermediates (50–52). In the multi-stress model (acidic pH, reactive nitrogen intermediates, hypoxia, and a fatty acid carbon source), some compounds specifically required reactive nitrogen intermediates for their activity (28, 53). Grant et al. found that of 52 molecules active against *M. tuberculosis* in a carbon starvation model, only 33% were also active against bacilli rendered nonreplicating by hypoxia (54). The same study found diversity of the activity profiles of 4 compounds, from 3 chemical classes, in a class I persister model and three class II models: hypoxia, starvation, and removal of streptomycin from the addicted strain, SS18b (54, 55).

Very few compounds have been demonstrated to kill *M. tuberculosis* rendered nonreplicating in more than one way. This may be because such “pan-actives” are rare in chemical space or because investigators do not routinely test actives from one model in other models. We describe pan-actives in the section “*Proof of concept molecules*”.

4. Class I persisters: rare, drug tolerant cells

Long before the work of Bigger, Hobby and colleagues was rediscovered (3, 4), and the term “phenotypic tolerance” became widely used (56), researchers had observed evidence of class I persisters *in vitro* and *in vivo*. Kill curves, in which the X-axis of the graph represents time, and the Y-axis represents the number of viable bacteria recovered on agar plates using a CFU-based assay, often have a biphasic, or “hockey stick”, shape (25). Following a sharp, logarithmic decrease in viable CFU at early time points, the CFUs plateau, or decrease at a reduced rate. Notably, compounds fail to reduce CFU below the limit of detection at any concentration tested (46, 55). Put differently, the CFU assay reveals a small population of cells that is refractory to killing by the antibiotic. Biphasic kill curves have been observed for *M. tuberculosis* and other mycobacterial species treated with dapsone, ciprofloxacin, isoniazid, D-cycloserine, rifampicin, streptomycin, and various combinations of these antibiotics (30, 46, 54, 55, 57, 58). Class I persisters appear to play a role in phenotypic drug tolerance during human infections caused by *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans* (57, 59–61). Evidence of class I persisters was observed in murine and guinea pig models of tuberculosis (62–64) and in the human disease (57).

By definition, class I mycobacterial persisters are reversibly tolerant to one or another of the standard antibiotics (46, 55, 65) but not necessarily to their combinations. There is no reason to expect class I phenotypically tolerant bacteria to be more resistant than their siblings to molecules that have multiple targets, such as hydroxyl radicals (55, 66). Unlike in *E. coli* (67), there is conflicting evidence whether class I mycobacterial persisters are cross-tolerant to other antibiotics. In one study, *M. smegmatis* and *M. tuberculosis* persisters that survived exposure to a combination of ciprofloxacin and isoniazid were tolerant to a bactericidal concentration of rifampicin (55). However, a different study found persister populations of $\sim 1.7 \times 10^{-5}$ to isoniazid, $\sim 7.0 \times 10^{-4}$ to rifampicin, and $> 10^{-1}$ to pyrazinamide (65). The persister population resistant to the combination of isoniazid, rifampicin, and pyrazinamide was $\sim 2.8 \times 10^{-7}$, indicating that individual persisters were not broadly resistant to other antibiotics (65). While strategies to target class I persisters have been proposed (40, 41, 68), to our knowledge, high throughput screens targeting class I mycobacterial persisters have not been undertaken. Conversely, most compounds known to have activity against mycobacteria have not been tested for activity against mycobacteria displaying class I phenotypic tolerance to other compounds. **Compound 57**, identified in a class II phenotypic screen against carbon-starved *M. tuberculosis*, serves as an illustrative example of a compound whose ability to additionally kill class I phenotypically tolerant *M. tuberculosis* was discovered post-screening (54). **Compound 57** is described in more detail in the section “*Screening hits: carbon starvation*”(54).

In vitro, genetic mutations in *hipA* and *hipB* (**high** persister genes) lead to approximately 10–10,000-fold more class I drug tolerant persisters in *E. coli*, *Salmonella*, and other species

(57, 69–73). Use of *hip* mutants permitted the observation of persisters by time-lapse studies in microfluidic devices (39). High persister mutants that survived treatment with streptomycin and rifampicin were recently identified in an ethyl methanesulfonate-mutagenized auxotrophic strain of *M. tuberculosis*, and characterized by genome re-sequencing and transcriptomics (57). Genetic control over the size of a class I phenotypically tolerant population should not be confused with heritable resistance. The survivors, when grown up without antibiotic and exposed again, have the same MIC as the population from which they were derived. Even a mutant strain of *E. coli* with a 10,000-fold increase in the wild type proportion of class I phenotypically tolerant persisters to ampicillin will display a 99% reduction in survival at the same concentration of ampicillin as the wild type strain if the proportion of class I persisters has increased from 1×10^{-6} to 1×10^{-2} .

Numerous mechanisms can impel a cell to display class I phenotypic tolerance (39, 74–79). Mycobacterial asymmetric division results in differential antibiotic sensitivity of daughter cells (80, 81). As in *E. coli* (82, 83), mycobacteria may depend on toxin-antitoxin genes (46, 65, 84) to induce class I tolerance. Javid and colleagues found that mistranslation of two amino acids, glutamate for glutamine, and aspartate for asparagine, resulted in modified RNA polymerase (RpoB, encoded by *rv0667*) that was more resistant to rifampicin (85). Only a minority of cells in a wild type population accumulated enough mutant copies of RpoB with Asp in place of Asn at position 434 to survive rifampin at its MIC (85, 86). When these cells were expanded, the MIC remained the same. Analogous to the situation with *hip* genes in *E. coli*, mutation in the GatCAB aminotransferase that normally corrects mistranslation of the Asn codon increased the frequency of these class I phenotypically tolerant mycobacteria, but the MIC was no greater in progeny of these cells than in the population from which they were selected (86). Rendering *M. smegmatis* nonreplicating by acidic pH or nutrient starvation led to protein mistranslation and phenotypic kanamycin resistance (85). Isoniazid is a pro-drug that requires oxidation by a catalase-peroxidase (KatG, encoded by *rv1908c*) and forms an NAD-isoniazid adduct that targets NADH-dependent enoyl-ACP reductase (InhA, encoded by *rv1484*). Isoniazid kills multiple $\log_{10}$ CFU of replicating mycobacteria within days; yet, isoniazid dosed by itself takes weeks to months to achieve a modest reduction in the *M. tuberculosis* bacterial burden in mice (87). Stochastic gene expression has been described in eukaryotes and prokaryotes (88, 89) and provides one potential explanation for the appearance of class I phenotypic tolerance in a small subpopulation of bacteria. For example, Wakamoto et al. found that stochastic expression of *katG* explains some mycobacterial tolerance to isoniazid (90). In *E. coli*, fluoroquinolones can damage DNA and induce an SOS response protein, TisB, which transforms cells to a persister phenotype by depolarizing the membrane and depleting ATP (91–93).

5. Class II persisters: a majority population of nonreplicating, drug tolerant cells

Class II persisters are defined as a population of cells displaying phenotypic drug tolerance under externally applied conditions that halt net replication. As noted, nonreplication in this sense is a terminologic simplification that encompasses balanced bacterial growth and death.

In some models of nonreplication, there is a slow reduction in viable bacteria over the period of observation that may be difficult to detect by a CFU assay (28, 94). Conditions that arrest growth are associated with resistance to a large number of antibiotics. Some investigators have assumed that failure to grow is synonymous with shutdown of the bacterial machinery that synthesizes macromolecules and that lack of need for macromolecules explains lack of sensitivity to drugs that inhibit their synthesis (30, 50–52, 95, 96). However, *M. tuberculosis* adapts to the stresses that impose nonreplication with a robust transcriptional response (46, 49, 97) and cell wall remodeling (98, 99) and maintains metabolic activity, although with a different profile of metabolites than during replication (K. Rhee, personal communication). Non-reliance on biosynthetic processes is an unsatisfactory explanation for class II phenotypic tolerance.

The rate at which *M. tuberculosis* achieves stasis may impact the bacilli's biology and sensitivity to certain compounds. For example, some models of nonreplication, such as hypoxia or starvation, require pre-adaptation periods of 1–2 weeks or more (12, 54, 95). In contrast, reactive nitrogen intermediates cause immediate growth arrest (48, 100). In addition, exogenously applied stresses may be perceived at different rates by mycobacteria at different locations within a clump.

5.1 Designing high-throughput screens to target phenotypically tolerant mycobacteria

There have been numerous whole cell screens to identify small molecules in academic and industrial collections that kill nonreplicating mycobacteria (29, 53, 54, 94, 96, 101–105). Compounds arising from whole cell screening are presumably taken up into the cell to exert bactericidal activity, without any preconceptions about suitable targets. An alternate approach is to postulate which enzymes play a role in nonreplicating persistence based on informatics or biochemical or genetic studies, set up relevant biochemical assays, identify inhibitors, and then assay these inhibitors for whole-cell activity in nonreplicating models (100, 106–115). The limitation of biochemical screening, however, is that the majority of enzyme inhibitors so identified lack activity against intact *M. tuberculosis* due to poor uptake, the sufficiency of residual enzyme activity for cell survival, intracellular metabolism, or redundant pathways (116). Translating biochemical screening hits to whole cell activity is hampered by using a binary readout of life/death of a bacterial cell as a surrogate to monitor target engagement (117).

In screens carried out against nonreplicating bacteria, a failure to increase in optical density over time cannot be used as a measure of anti-bacterial activity since, by definition, the optical density does not change for the duration of a nonreplicating experiment. There are limited examples of screening by recording fluorescence from nonreplicating mycobacteria (29, 118, 119). While most replicating assays use an inoculum of $\sim A_{580}$ of 0.01 or lower, use of a fluorescent readout can require a larger inoculum (upwards of ~ 50 -fold) to achieve a sufficient signal (120). Using a high inoculum of cells may preclude identifying active molecules from compound classes such as beta-lactams, which are highly sensitive to inoculum effects (121). Moreover, nonreplicating screens employing fluorescent readouts often depend on subtle differences in the fluorescence of compound-treated versus vehicle-treated cells (often less than 2-fold), which in turn requires exceptional Z' scores (29). In

some nonreplicating assays, the drug-exposure phase of the assay is coupled to a drug-free secondary phase that permits bacterial growth, and allows one to make a semi-quantitative estimation of the number of surviving cells (Figure 1) (53, 54, 96). The two-stage assay, while effective, can take 14 to 17 days (a 7-day drug exposure, and a 7–10 day outgrowth), and runs a risk of evaporation, edge effects, and contamination with mold (30, 94).

One must carefully weigh the relative importance of potential variables when designing a high-throughput screen against nonreplicating mycobacteria (Figure 2). Table 2 provides a non-exhaustive list of potential microenvironments encountered by *M. tuberculosis* during infections that may lead to sub-optimal growth or nonreplication. The numerous nonreplicating models and technical variables lead to a staggering number of possible combinations.

The most commonly used models for nonreplicating mycobacteria are: hypoxia (the “Wayne model”) and the “low oxygen recovery assay” (“LORA”) (29, 95, 96); carbon starvation (54, 122); nutrient starvation (12, 52); stationary phase (105); maintenance of intrabacterial pH under acidic culture conditions (120, 123–127); biofilms (102, 128–131); depleting strain SS18b of streptomycin (103, 104, 132, 133); and a multi-stress model that combines acidic pH (pH 5.0), mild hypoxia (1% O₂), nitric oxide and other reactive nitrogen intermediates (0.5 mM NaNO₂), and a fatty acid carbon source (0.05% butyrate) (53, 94, 100, 134, 135). There are variations of these models, including an “acidic Wayne model” that combines hypoxia with mild acidity (131), and a nutrient-poor, multi-stress model in which cells are cultured at low pH (pH 5.0) under mild hypoxia or tissue-level normoxia (5% O₂) and supra-physiologic levels of CO₂ (10% CO₂) (136). Sub-lethal doses of antibiotics targeting translation can also arrest growth (137). Potassium starvation has been reported to lead to the formation of differentially detectable mycobacteria (also called, “viable but not culturable”) (138, 139).

High throughput screening typically identifies many molecules with properties unsuitable for further progression, including those whose structures contain toxicophores and/or metabolic liabilities (140, 141). Comprehensive post-screening characterization of compounds from primary screens is extremely expensive in terms of time and resources. In Table 3, we summarize post-screening assays that are suitable for molecules with activity against replicating and/or nonreplicating mycobacteria. Table 4 summarizes assays used to characterize the action of compounds on nonreplicating mycobacteria. A hit progression flowchart for a nonreplicating active compound should attempt to include the assays described in both Tables 3 and 4.

5.2 Potential compound transformation during screening and secondary assays

As molecules progress from the initial high-throughput screen to *in vivo* models, there are mounting risks of wasting progressively larger amounts of time and money. Meticulous analysis of physicochemical and metabolic properties (140) of compounds is the norm in pharmaceutical companies, and unfortunately, often pursued insufficiently in academia due to lack of experience, personnel, funds, or access to expert chemistry advice, experimental analysis and synthesis (141).

Chemical structures can be misidentified as a result of inaccurate assembly of the compound library, erroneous dispensing of compounds, incorrect structure assignment, splashing of compounds between microtiter wells, compound degradation, and incomplete removal of reagents or catalysts used to synthesize the original compound, such as organotin, which can have antiseptic properties (142). For these reasons, it is critical to validate a molecule's structure after cherry-pick confirmation and prior to initiating downstream hit characterization. Validation studies include testing a subset of screening hits for correct molecular mass, structure and purity by LC-MS and NMR. Prioritized molecules should be re-synthesized and re-evaluated in the original assay to confirm that they recapitulate the activity of the original hits. A surprisingly large number of screening compounds fail to meet these criteria.

Compound solubility is a problem at the forefront of high-throughput screening. The real and assumed concentrations of drug stocks can differ by several orders of magnitude (143–145). DMSO is hygroscopic and can absorb water from room air, leading to compound precipitation of water-insoluble compounds. Some DMSO-soluble compounds precipitate immediately, or over time, when transferred to assay plates containing aqueous media or buffers. Antimycobacterial compounds often have high logP values (146) that favor their precipitation in aqueous solution. Compound precipitation can lead to false-negative activity or to false-positive activity in optical density-based assays.

As scientists explore more diverse bacteriologic media for whole-cell screening to mimic *in vivo* microenvironments and stresses, another issue arises – the chemical stability of the compounds in the assay conditions. Careful determination of the structure of a molecule under the nonreplicating assay conditions is a critical, and often overlooked, step. As illustrated in Figure 3a, structures may be transformed by conditions found in nonreplicating assays, including acidic pH, reactive oxygen intermediates and reactive nitrogen species. If specific transformation products can be identified, they should be tested for activity in the original model of nonreplication and for their potential toxicity to eukaryotic cells. For example, oxyphenbutazone was chemically transformed in cell-free medium used in the multi-stress assay of nonreplication (Figure 3b) (53). In acidic medium containing reactive nitrogen species, the carbon on which the butyl chain attaches to the pyrazolidinedione ring was hydroxylated to form 4-hydroxy-oxyphenbutazone. This oxidation was followed by the pyrazolidinedione ring opening and formation of a quinoneimine. 4-Hydroxy-oxyphenbutazone's quinoneimine, a Michael acceptor, reacted *in vitro* with glutathione and mycothiol (Figure 3b). In live *M. tuberculosis*, intracellular covalent adducts formed between 4-hydroxy-oxyphenbutazone and mycothiol, N-acetyl cysteine, and other uncharacterized metabolites. 4-Hydroxy-oxyphenbutazone killed replicating *M. tuberculosis* and mediated some of oxyphenbutazone's activity against nonreplicating mycobacteria. In another example, three cephalosporin analogues (one of which was **compound 68**) with equipotent activity against *M. tuberculosis* rendered nonreplicating in the multi-stress model had different stability profiles: 2 were stable, and one was unstable (147). These results suggested that their uptake into *M. tuberculosis* occurred more rapidly than their extracellular transformation. As this example indicates, the relevance of cell-free stability should be evaluated on a case-by-case basis.

5.3 Evaluating bactericidal action against nonreplicating mycobacteria

Replicating bacterial cultures fail to increase in biomass when treated with effective concentrations of either bacteriostatic or bactericidal molecules. This makes it relatively straightforward to recognize when compounds are active on replicating mycobacteria. Assays can be short in duration; there are many ways to assess viability; and false-positives are unlikely.

In contrast, determining the impact of compounds on nonreplicating cells is technically challenging. Nonreplicating conditions are themselves bacteriostatic, precluding the detection of viability using methods suitable for replicating cells. By coupling nonreplicating assays to a recovery phase under replicating conditions, one only obtains a rough estimation of a compound's activity (Figure 1) (28, 53, 54, 94, 96). In the case of dual active molecules, one must ensure *bona fide* activity against the nonreplicating cells, or, alternatively, determine if the nonreplicating activity is an artifact of compound carry-over from the nonreplicating phase of the assay into the replicating phase of the assay (Figure 1) (30). Carry-over need not be via the fluid phase; compounds can absorb to mycobacterial components and be carried over to the replicating phase of the assay (30). As noted, drug carry-over was shown to be particularly troublesome for the extremely potent and extremely hydrophobic compound, TMC207 (30, 32, 148). This is not to deny that TMC207 has utility in animal (148, 149) and human (150) tuberculosis, and in fact, drug adsorption to mycobacteria may be a useful property. For example, compounds that associate with the bacterial cell wall may deliver a potent post-antibiotic effect (30) as they are slowly released into the intrabacterial cytosol. *In vitro* assays have arbitrary time points that are far shorter than clinical regimens and as such may grossly underestimate a drug's bactericidal potential.

We and others have attempted to minimize carry-over effects from enumerating bacilli from *in vitro* assays or from organs harvested from antibiotic-treated, *M. tuberculosis*-infected animals (31–33). One solution was to include 0.4% (w/v) activated charcoal in bacteriologic agar plates to rapidly and completely bind the majority of first and second line anti-mycobacterial antibiotics (30).

Many compounds with nonreplicating activity were originally identified as highly potent replicating actives. Only a few studies confirmed their nonreplicating activity with a CFU assay, and almost never in the presence of activated charcoal or BSA in the agar plates. Given the challenge of testing large numbers of candidate dual active molecules by the CFU assay, the charcoal agar resazurin assay (CARA) was developed to rapidly categorize molecules as replicating-bacteriostatic, replicating-bactericidal, nonreplicating-bactericidal, or dual-active (that is, replicating bacteriostatic or bactericidal and nonreplicating bactericidal) (30). The CARA helps indicate which compounds should be explored by CFU assays and at which concentrations.

5.4 Killing class II persisters

Molecules that reportedly kill class II phenotypically tolerant mycobacteria are structurally diverse. We have grouped approximately 100 such compounds according to core structure, targets, and/or method of discovery (Figures 4, 6–11, and 13).

The list of compounds was assembled with the intent of demonstrating both diversity and common themes. However, this is by no means a complete catalog. Additional actives can be found in databases such as SciFinder (<https://scifinder.cas.org>), PubChem (<https://pubchem.ncbi.nlm.nih.gov/>), and Collaborative Drug Discovery (<https://www.collaborativedrug.com/>) (151). Many other actives found in screening do not have their structures in scientific reports or deposited in public databases.

A relatively small number of compounds described to have bacteriostatic or bactericidal activity against replicating mycobacteria have also been tested for activity against nonreplicating bacteria. At best, most compounds were tested against mycobacteria in a single model of nonreplication. Many studies did not test the activity of the reported compounds with the gold standard CFU-based assay to determine viability.

Highly potent replicating actives may register as false-positives in nonreplicating assays due to compound carry-over from the nonreplicating phase to a replicating phase (see section “*Evaluating bactericidal action against nonreplicating mycobacteria*”). TMC207, which is active in multiple nonreplicating models, is an example of compound that has a high propensity for carry-over, clouding interpretation of results (30, 152). Some molecules are listed under more than one classification. For example, TMC207 is in three figures depicting structures: “*Proof-of-concept molecules*”, “*Quinolines*”, and “*Membrane depolarizers*”.

The distinction between bactericidal and bacteriostatic varies significantly in the literature. For this review, we define bacteriostatic as < 99% bacterial kill (< 2 log₁₀ CFU) in 15 days (30, 153).

5.4.1 Proof of concept molecules—A major hurdle for large-scale commitment of resources towards identifying compounds that target nonreplicating bacteria is the paucity of examples that demonstrate the success of this approach. ADEP4 (**compound 48**), a synthetic acyldepsipeptide that dysregulates ClpP proteolysis, may serve as a prototype (154). ADEP4 killed both *S. aureus* class I persisters surviving ciprofloxacin exposure, and bacteria in three class II models of nonreplication: stationary phase, chemically defined minimal medium, and biofilms (155). ADEP4, when dosed with rifampicin, eradicated *S. aureus* in a mouse thigh infection. It is unknown to what extent the activity of ADEP4 against the replicating and nonreplicating populations contributes to its *in vivo* efficacy (155). In the following sections we explore examples of proof of concept molecules in mycobacteria.

5.4.1.1 Selective nonreplicating activity: There are few examples of molecules that fail to kill replicating mycobacteria, and kill those rendered nonreplicating in one or more *in vitro* models of nonreplication (Figure 4a). Although a limited number of molecules with pan-activity against nonreplicating mycobacteria have been identified, it is likely that more would emerge if the appropriate tests were performed. Since there is a paucity of common transcriptional responses among *M. tuberculosis* populations rendered nonreplicating in different *in vitro* models, such experiments are critical. The first example of a molecule with pan-activity against nonreplicating mycobacteria was published in 2008 (100). Bryk et al. identified a rhodanine, D157070 (**compound 1**) from a structure activity relationship campaign to develop a pro-drug inhibitor of dihydrolipoamide acyltransferase, DltA (100,

156, 157). D157070 selectively killed *M. bovis* BCG and *M. tuberculosis* rendered nonreplicating by acidic pH and reactive nitrogen intermediates (34), hypoxia (95), a multi-stress model of nonreplication combining acidic pH, reactive nitrogen intermediates, hypoxia and restriction of the carbon source to a fatty acid (53, 94), human tissue culture medium (Dulbecco's modified Eagle medium containing 10% FBS), and infection of bone marrow derived macrophages activated with IFN γ (100, 158).

5.4.1.2 Dual actives with in vivo efficacy: Dual active molecules are defined as possessing bacteriostatic or bactericidal activity against replicating bacteria and bactericidal activity against nonreplicating bacteria. Of the dual actives, moxifloxacin (**compound 2** (30, 50, 51, 96, 132, 159), PA-824 (**compound 3** (30, 50, 51, 96, 132, 152, 160), rifampicin (**compound 4** (30, 50, 51, 95, 96, 132, 152, 159–164) and TCM207 (**compound 5**, bedaquiline (30, 50, 51, 132, 150, 152, 159, 165, 166) are reported to kill mycobacteria rendered nonreplicating in diverse ways, including hypoxia, nutrient starvation, stationary phase, multi-stress, and deprivation of streptomycin from an addicted strain (Figure 4b). Moxifloxacin, PA-824, rifampicin and TMC207 target DNA gyrase, lipid/protein biosynthesis, RNA polymerase, and ATP synthase, respectively.

While none of the available *in vitro* or *in vivo* assays (including those described in Tables 3 and 4) can predict the efficacy of a compound in human tuberculosis, it is reasonable to prioritize compounds that have dual activity and potency against mycobacteria infecting macrophages and mice. In fact, a combination of three compounds in Figure 4b, PA-824, moxifloxacin, and PZA (PaMZ), showed promise in the NC001 clinical trial in tuberculosis patients (167). The 14-day NC001 EBA study was too brief to evaluate the impact of PaMZ on eradicating persisters and decreasing relapse rates (167). The Nix-TB clinical trial is evaluating the combination of TMC207, PZA, and linezolid on MDR- and XDR-tuberculosis, and may shed light on this question by increasing the duration of treatment up to 6–9 months (<http://www.tballiance.org/portfolio/trials>).

5.4.1.3 Nonreplicating actives with in vivo efficacy: Of the molecules with selective activity against slowly replicating or nonreplicating mycobacteria, only metronidazole (**compound 6**) (95) and pyrazinamide (**compound 7**) (168) have been shown to be effective in animal models of tuberculosis (Figure 4c). As described in the introduction, metronidazole is bactericidal to hypoxic mycobacteria but this was only demonstrated under conditions in which no alternate electron acceptor was provided; killing was attenuated by inclusion of nitrate, a physiologic electron acceptor used by hypoxic *M. tuberculosis* and present in body fluids (169, 170). Activity of metronidazole did not correlate with evidence of lesion hypoxia (Table 1). Pyrazinamide is the sole representative of the nonreplicating-active class of compounds that is known to kill *M. tuberculosis* in humans, but pyrazinamide also kills slowly replicating mycobacteria *in vitro*. Pyrazinamide's activity on *M. tuberculosis* under acidic conditions was enhanced by additionally including hypoxia (23) or a 3–10 day period of pre-adaptation to nutrient starvation in PBS (171). While its complete set of targets is still under investigation, pyrazinamide inhibits fatty acid biosynthesis by targeting FAS-I (172–174), trans-translation by targeting ribosomal protein S1 (RpsA,

encoded by *rv1630* (175) and pantothenate and coenzyme A by targeting aspartate decarboxylase (PanD, encoded by *rv2601c*) (176, 177).

5.4.2 Molecules anticipated to engage canonical antibiotic targets and pathways—

The potency of many dual active molecules in Figure 4 was lower against nonreplicating bacilli than against mycobacteria replicating in standard bacteriologic medium. This could be due to less reliance on these processes during nonreplication, decreased uptake of the compounds due to a change in membrane composition and/or permeability (178), compound modification by the assay conditions (Figure 3) (28, 53), increased or altered intrabacterial metabolism of the compound (116), or sequestration of compound into lipid bodies that accumulate in mycobacteria in some in vitro models of nonreplication (28, 53, 136). Moreover, dual actives may kill nonreplicating mycobacteria by engaging non-canonical targets, or have a non-specific mechanism of action (Figure 5).

The canonical targets of many compounds that kill nonreplicating mycobacteria are in pathways for the biosynthesis of cell wall, lipids, RNA, DNA, protein or peptidoglycan (Figure 6). These compounds build a compelling case that nonreplicating mycobacteria engage in turnover of macromolecules. It is particularly encouraging that numerous antibiotic classes, some of whose members are approved for use in humans, including fluoroquinolones, rifamycins, macrolides, tetracyclines, and beta-lactams, have representatives that kill nonreplicating *M. tuberculosis*. This suggests that the anti-mycobacterial members of these families may likewise be tailored to display the pharmacokinetic and pharmacodynamic properties and low toxicities that allowed approval of the family members in clinical use (179).

Compounds that generate reactive oxygen species or reactive nitrogen species likely impact the function of numerous targets, including lipids, DNA, and the membrane. For example, PA-824 donates reactive nitrogen species (160). Sub-lethal nitric oxide induced a specific set of genes in the *dos* regulon, but higher concentrations of nitric oxide induced the expression of hundreds of other genes and implicated reactive nitrogen species in interfering with numerous processes (48, 180). In addition to engaging high affinity targets, compounds like PA-824 are probably promiscuous when they achieve higher intrabacterial concentrations.

High-affinity targets may exist that have an essential function unique to mycobacteria in a nonreplicating state. However, to date, we know of no instance in which differential expression or differential essentiality of a target has been shown to explain how a compound selectively kills nonreplicating mycobacteria.

5.4.2.1 Lipid synthesis: A tetrahydrobenzothienopyrimidine (**compound 8**) targeting InhA killed *M. tuberculosis* rendered nonreplicating by hypoxia (181) (Figure 6a). That InhA might be an essential target during hypoxia is surprising. Isoniazid, which targets InhA, does not kill hypoxic *M. tuberculosis* or *M. tuberculosis* rendered nonreplicating in other conditions. Isoniazid is even used experimentally as a control compound to confirm that cells have achieved a state of nonreplication. This raises an important question why isoniazid fails to kill hypoxic, nonreplicating mycobacteria. The structure of isoniazid (likely the hydrazide moiety) may be unstable in hypoxia and/or other nonreplicating conditions

(Figure 3a). Perhaps KatG fails to activate isoniazid under nonreplicating conditions. To test this hypothesis, InhA inhibitors that do not require KatG activation could be tested against nonreplicating bacilli (182). Another possibility is that **compound 8**, like isoniazid itself, may have more than one target, but unlike isoniazid, one of the alternate targets of **compound 8** may be essential in hypoxia.

PA-824 is an inhibitor of lipid and protein synthesis (183, 184). While it has multiple targets, pyrazinamide is an inhibitor of lipid biosynthesis (172–174). Both PA-824 and pyrazinamide are described in the section, “*Proof of concept molecules*”.

5.4.2.2 DNA synthesis: Nonreplicating conditions may lead to oxidative stress, as may antibiotics with diverse primary targets (55, 66). DNA damage may result and survival may require DNA repair. Compounds that target DNA synthesis and kill nonreplicating mycobacteria are shown in Figure 6b. Inhibitors of topoisomerase I (TopA, encoded by *rv3646*) (**compound 9**) (113) and DNA gyrase B (GyrB, encoded by *rv0005*) (**compounds 10 and 11**) (108, 112) killed *M. tuberculosis* rendered nonreplicating by nutrient starvation, redox stress or hypoxia. Cyclohexyl griselimycin (**compound 12**), which targets the DnaN (encoded by *rv0002*) sliding clamp of DNA polymerase, is anticipated to kill nonreplicating *M. tuberculosis* due to its ability to reduce CFU during the persistent phase of murine tuberculosis (185–187). To our knowledge, the activity of cyclohexyl griselimycin against *M. tuberculosis* rendered nonreplicating by *in vitro* models has not been explored. Multiple fluoroquinolones, whose canonical targets are DNA gyrase and/or topoisomerase IV (188), including ciprofloxacin (**compound 13**) (51), gatifloxacin (**compound 14**) (51), levofloxacin (**compound 15**) (51), moxifloxacin (30, 50, 51, 96, 132, 159), and sparflaxacin (**compound 16**) (51), killed nonreplicating *M. tuberculosis* also described in the section “*Quinolones*”).

5.4.2.3 RNA synthesis: The transcriptomic adaptations of mycobacteria in nonreplicating conditions imply a requirement for RNA synthesis for their survival. Inhibitors of RNA polymerase (RpoB, encoded by *rv0667*) (Figure 6c), including rifampicin (described in “*proof of concept molecules*”) (30, 50, 51, 95, 96, 132, 152, 159–161), rifabutin (**compound 17**) and rifapentine (**compound 18**), killed nutrient starved and hypoxic *M. tuberculosis* (51, 96).

5.4.2.4 Protein synthesis: Mycobacteria are killed by a large number of compounds belonging to different structural classes and targeting diverse steps in protein biosynthesis (Figure 6d). The newly synthesized proteins may help mycobacteria detoxify or compensate for the stresses imposed by nonreplication. In mycobacteria, protein translation is vastly decreased, but not abrogated, during the first 40 days of nonreplication (189). Numerous compounds targeting the 30S and 50S components of the ribosomal machinery killed *M. tuberculosis* in multiple models of nonreplication, including deprivation of strain SS18b for streptomycin, hypoxia, and nutrient starvation. The protein synthesis inhibitors included the oxazolidinones linezolid (**compound 19**) (103) and sutezolid (**compound 20**) (103); the tetracycline minocycline (**compound 21**) (96); the aminoglycosides amikacin (**compound 22**) (51, 96), streptomycin (**compound 23**) (50, 51, 96), and kanamycin (**compound 24**)

(51); an aminocyclitol antibiotic, the spectinomycin analogue 1599 (**compound 25**) (190); the cyclic peptide antibiotic capreomycin (**compound 26**) (50, 51, 96); the quinoline macrolide RU66252 (**compound 27**) (96); and fusidic acid, which prevents elongation factor G turnover and translocation (**compound 28**) (96). *M. tuberculosis* rendered nonreplicating by incubation for 2 months in stationary phase and then acidified to pH 5.5 under mild hypoxia (1% O₂) was susceptible to methionine aminopeptidase inhibitors (**compounds 29** (114, 115) and **30** (114)). PA-824, previously described as disrupting lipid biosynthesis, was also shown to inhibit protein synthesis (183).

5.4.2.5 Peptidoglycan synthesis: Until recently, the dogma in the tuberculosis field was that *M. tuberculosis* was naturally resistant to beta-lactams. The two leading hypotheses were that beta-lactams failed to cross the mycobacterial outer membrane, and that beta-lactams were susceptible to beta-lactamase cleavage (191–195). Unexpectedly, there are now multiple examples of beta-lactams and other molecules targeting steps in peptidoglycan biosynthesis that kill nonreplicating mycobacteria (Figure 6e).

The canonical targets of beta-lactam antibiotics are enzymes catalyzing steps in peptidoglycan biosynthesis. The correlation between the bacterial replication rate and beta-lactam activity fostered the assumption that beta-lactams selectively target replicating bacteria (196). The choice of compounds for these studies led to the belief that activity was restricted to replicating cells, although some beta-lactams were identified that killed both replicating and nonreplicating *Streptococcus pneumoniae* and *E. coli* (56). The basis of dual activity remained a mystery for many years. Most bacteria contain murein predominantly composed of 4 → 3 transpeptides (197, 198). *M. tuberculosis* in stationary phase, or in hypoxia, had peptidoglycan enriched for 80% and 68% 3 → 3 crosslinks, respectively (98, 99). These studies suggest that the peptidoglycan layer in nonreplicating cells may be different than that of replicating cells, and if so, offers an underexplored set of target enzymes, such as the L,D-transpeptidases (199). A caveat to this conclusion, however, is that 3 → 3 crosslinks were also enriched in replicating *M. tuberculosis* (~ 62%) and may not be unique to nonreplicating mycobacteria (99).

A landmark paper in 2009 by Hugonnet et al. demonstrated that meropenem (**compound 31**), when paired the beta-lactamase inhibitor clavulanic acid (**compound 32**), killed replicating *M. tuberculosis* (195). The Hugonnet study made a critical, and unanticipated, discovery – that the combination of meropenem and clavulanate also killed hypoxic, nonreplicating *M. tuberculosis* (195). A 14-day trial demonstrated that meropenem, amoxicillin and clavulanate had marked early bactericidal activity in human tuberculosis (200).

A number of other molecules have been reported to target nonreplicating mycobacteria by disrupting steps of peptidoglycan biosynthesis, including: L,D-transpeptidases by faropenem (**compound 33**) (38, 201); UDP-galactopyranose mutase (**compound 34**) (202); phospho-N-acetylmuramoyl-pentapeptidetransferase (MurX) by CPZEN-45 (**compound 35**) (203); and the capuramycin analogue UT-01320 (**compound 36**) (204). CPZEN-45 may additionally target decaprenyl-phosphate-GlcNAc-1-transferase (WecA, encoded by *rv1302*), which has a role in synthesizing teichoic acid in *Bacillus subtilis* and mycoylarabinogalactan in

mycobacteria (205). UT-01320 (**compound 36**) was bactericidal to both hypoxic and nutrient-starved *M. tuberculosis* (204). The structure of capuramycin (**compound 37**) illustrates an example in which replacing a hydroxyl group with an O-methyl (UT-01320, yellow highlighted carbon atom) confers nonreplicating activity on a molecule whose activity was restricted to replicating mycobacteria. However, UT-01320's activity profile change was accompanied by the failure to inhibit MurX *in vitro* and suggests that its ability to kill nonreplicating *M. tuberculosis* may have been due to engaging a different target (204).

5.4.2.6 Folate synthesis: We did not find reports of nonreplicating mycobacteria being killed by inhibitors of folate biosynthesis.

5.4.3 Quinolines and their derivatives—There are numerous examples of quinolines and their derivatives that kill nonreplicating mycobacteria (Figure 7).

5.4.3.1 Quinolines: Maintaining ATP levels is critical for mycobacteria to survive the nonreplicating state (166). TMC207, which has a quinoline core, is bacteriostatic to replicating mycobacteria, and has been reported to kill those rendered nonreplicating by hypoxia, nutrient starvation, and streptomycin removal from the addicted strain SS18b (30, 50, 51, 132, 150, 152, 159, 165, 166). Due to its hydrophobic nature (logP of 7.3) and nanomolar potency, TMC207 is subject to carry-over artefacts that make it challenging to evaluate its activity in nonreplicating models (30). To our knowledge, TMC207's activity against mycobacteria in nonreplicating models has not been established by CFU enumeration under conditions that prevent drug carry-over, such as the presence of 0.4% (w/v) activated charcoal in the bacteriologic agar (described in “*Evaluating bactericidal action against nonreplicating mycobacteria*”). In our own studies, use of activated charcoal eliminated most of the apparent activity of TMC207 against *M. tuberculosis* in a multi-stress model of nonreplication (30).

Another ATP synthase inhibitor, the substituted chloroquinoline **compound 38** (161, 206) was reported to be bactericidal to hypoxic *M. tuberculosis*. The antimalarial drug mefloquine (**compound 39**) was reported to kill *M. tuberculosis* rendered nonreplicating by hypoxia and nutrient starvation (51). Mefloquine targets ATP synthase in *Streptococcus pneumoniae* (207, 208), and its target in *M. tuberculosis* is currently not known.

5.4.3.2 8-hydroxyquinolines: 8-hydroxyquinolines have antibacterial activities on *M. tuberculosis in vitro* and *in vivo* (209, 210). The unsubstituted 8-hydroxyquinoline (**compound 40**) killed replicating *M. tuberculosis* and was bactericidal to *M. tuberculosis* rendered nonreplicating by mild hypoxia (1% O₂), acid (pH 5.5), and acidic nitrosative conditions (pH 5.5 containing 0.5 mM NaNO₂) (101). **Compound 30**, a 5-chloro, 7-bromo-substituted 8-hydroxyquinoline inhibitor of methionine aminopeptidase, killed *M. tuberculosis* that had been in stationary phase for two months (114). Some 8-hydroxyquinolines may mediate toxicity by chelating metals essential for mycobacterial survival, or by forming metal-quinoline complexes that engage target enzymes (211).

5.4.3.3 4-hydroxyquinolines: Phenotypic screening led to the discovery of mefloquine-like molecules with a 4-hydroxyquinoline core (**compounds 41 and 42**) that killed both replicating and hypoxic, nonreplicating *M. tuberculosis* (212–215). Nontoxic isoxazolecarboxylic acid ethyl esters analogues of **Compounds 41 and 42**, represented by **compound 43**, killed replicating and nonreplicating hypoxic *M. tuberculosis* (215, 216). 2-substituted 4-hydroxyquinolines (**compounds 44 and 45**) inhibitors of triacylglycerol lipase LipY (encoded by *rv3097c*) killed hypoxic *M. tuberculosis* (217). The activity of **compounds 44 and 45** was specific to recovery of dormant *M. tuberculosis*, in which LipY plays an essential role in triacylglycerol breakdown (218). With our colleagues at Sanofi, U. North Carolina, Memorial Sloan Kettering, and the Lankenau Institute, we identified and characterized 4-hydroxyquinolines with bactericidal activity against mycobacteria in the multi-stress model of nonreplication (data not shown).

5.4.4 Fluoroquinolones—Numerous fluoroquinolones kill nonreplicating *M. tuberculosis* (Figure 8). As mentioned, moxifloxacin kills mycobacteria in numerous nonreplicating models (30, 50, 51, 96, 132, 159). Similar to moxifloxacin, other fluoroquinolones possess activity against hypoxic, nonreplicating *M. tuberculosis*, including ciprofloxacin (51), gatifloxacin (51), levofloxacin (51) and sparfloxacin (51).

5.4.5 The proteolysis / proteostasis pathway—Inhibitors of the mycobacterial proteolysis and proteostasis pathways are selectively active against bacilli that are nonreplicating or have dual activity (Figure 9). In addition to turnover of undamaged proteins, the proteolysis pathway also degrades damaged or misfolded proteins that result from exposure to stresses such as reactive nitrogen intermediates or oxidative damage (55, 219). The proteostasis pathway helps insure proper folding of nascent, misfolded, or damaged proteins with the aid of chaperone proteins and refoldases (220). Some stresses that contribute to nonreplicating persistence engage the proteolysis and proteostasis pathways (Table 2) (55, 66, 219). As described previously, the acyldepsipeptide ADEP4 (**compound 49**) forces a bacterium to eat itself by unregulated digestion of intrabacterial proteins (155). Unlike the PrcBA proteasome (encoded by *rv2109c* and *rv2110c*), the ClpP1P2 proteolytic machinery (encoded by *rv2461c* and *rv2460c*) is essential for *M. tuberculosis* to survive under replicating conditions (221). The dual active molecules cyclomarin A (**compound 46**) (222), ecumicin (**compound 47**) (51, 132, 159, 166, 222, 223), and lassomycin (**compound 48**[cyclic(GLRRLFAD)]-QLVGRRNI-CO₂CH₃) (223–225), target the ClpP1P2 pathway by engaging ClpC, and kill *M. tuberculosis* rendered nonreplicating by hypoxia or stationary phase (222, 223, 225, 226). Ecumicin stimulates ClpC's ATPase and impairs its activator activity, while sparing ClpP1P2's proteolytic activity (223, 226). This results in depletion of cellular ATP (see, “*Membrane depolarizers*”). While not essential for logarithmic growth, the *M. tuberculosis* proteasome is required to survive stationary phase and long-term nutrient starvation (227, 228). Small molecule inhibitors targeting the proteasome, such as GL-5 (**compound 50**, an irreversible inhibitor) (106) and DPLG-2 (**compound 52**, a reversible inhibitor) (229), killed *M. tuberculosis* rendered nonreplicating with acidified nitrite. **Compound 51**, an analogue of GL-5, had bactericidal activity against a 2-week nutrient starved culture of *M. tuberculosis* (230).

These studies indicate that targeting the ClpP1P2 and PrcAB proteolysis pathways are good strategies to kill nonreplicating persisters.

5.4.6 Screening hits—There have been a limited number of phenotypic high-throughput screens to discover molecules that kill nonreplicating mycobacteria (Figure 10). The following non-exhaustive set of examples illustrates screening methods and the molecular diversity of the hits.

5.4.6.1 Carbon starvation: Grant et al. identified compounds that killed carbon-starved, nonreplicating *M. tuberculosis* (54) (Figure 10a). The screen was notable for testing a set of confirmed actives against *M. tuberculosis* in different class I and class II models of phenotypic tolerance. This strategy uncovered that the hits had diverse activities: some molecules were active only in the carbon starvation model used for the screen (**compounds 53 and 54**); others were active in carbon starvation and hypoxia models (**compounds 55, 56, and 57**; and **compound 58** was bactericidal to *M. tuberculosis* rendered nonreplicating by carbon starvation or hypoxia as well as to *M. tuberculosis* in a class I persister model.

5.4.6.2 Hypoxia: As noted, metronidazole kills hypoxic *M. tuberculosis in vitro* and can reduce CFU in some animal models of tuberculosis Table 1). Mak et al. set out to identify more molecules that killed nonreplicating, hypoxic *M. bovis* BCG (29) (Figure 10b). The hypoxia screen was performed using a recombinant *M. bovis* BCG strain expressing the *M. tuberculosis narGHJI* operon. This strategy artificially increased ATP levels by augmenting BCG's use of nitrate as an electron acceptor and made it easier to identify active molecules by their ability to decrease cellular ATP content. Active molecules of the benzimidazole, imidazopyridine and thiophene classes were confirmed by a CFU assay (**compounds 59, 60 and 61**).

5.4.6.3 Multiple physiological stresses: One strategy to identify molecules that kill nonreplicating mycobacteria is to combine various stresses encountered by *M. tuberculosis* during infections of macrophages, animals, and humans (49, 53, 125, 231–247). A combination of stresses is anticipated to capture a greater diversity of screening hits than a single stress. This strategy requires a secondary hit deconvolution to identify which stress(es) are essential for a given compound's activity. Our laboratory used this strategy to develop a multi-stress assay of nonreplication, in which *M. tuberculosis*'s replication was halted by mild acidity (pH 5.0), nitric oxide and other reactive nitrogen intermediates generated by NaNO₂ at acidic pH, a fatty acid carbon source (butyrate), and mild hypoxia (1% O₂). Examples of molecules active in the multi-stress model of nonreplication (Figure 10c) include **compounds 62, 63, 64, 65, and 66** (28), oxyphenbutazone (**compound 67**) (53), nitrofuranylcalanolide (**compound 81**) (134) (*described further in "nitro-containing compounds"*), and cephalosporins **68 and 69** (147). The cephalosporins, **compounds 68 and 69**, bearing a C-2 alkyl ester or oxadiazole had clavulanate-independent bactericidal activity against *M. tuberculosis* in a multi-stress model of nonreplication (147) and killed *M. tuberculosis* infecting IFN γ -activated bone marrow mouse macrophages. The unusual structures and activity profiles of **compounds 68 and 69** suggests that they may engage non-canonical targets (248–251).

5.4.6.4 Acidic pH: The intraphagosomal environment of an *M. tuberculosis*-infected, IFN γ -activated mouse macrophage decreases to approximately pH 4.5 (233). Furthermore, *M. tuberculosis* may create local acidic environments by secreting succinate when cultured under hypoxic conditions (22, 170). Using an intrabacterial pH-sensitive green fluorescent protein, Darby et al. screened for compounds that disrupted *M. tuberculosis*'s intrabacterial pH during incubation in growth-restricting conditions of phosphate-citrate pH 4.5 buffer (119, 123, 125) (Figure 10d). **Compounds 70, 71, 72, 40 and 73**, identified in this screen, had acidic-pH dependent bactericidal activity. High throughput antibacterial screens, in which bacteria are incubated in an acidic pH, often identify protonophores as nonspecific disrupters of the membrane proton gradient. Monensin (**compound 88** (120)) is one such example and is described in more detail in the section “*Membrane depolarizers*”.

5.4.6.5 Biofilms: Many pathogenic bacteria form biofilms as a survival strategy to evade host immunity and antibiotics (252, 253). Mycobacteria form drug tolerant biofilms *in vitro* (129, 254), although the relevance of mycobacterial biofilm formation in human disease is unknown. Wang et al. screened for compounds that killed nonreplicating *M. smegmatis* that presented as a pellicle at the interface of the bacteriologic medium and air. They identified a replicating-active molecule, TCA1 (**compound 74**) (Figure 10e), that potently inhibited biofilm formation by *M. smegmatis*, *M. tuberculosis*, and *M. bovis* BCG, and killed *M. tuberculosis* in a nutrient starvation model (102). TCA1 targets both decaprenyl-phosphoryl- β -D-ribose 2-oxidase (DprE1, encoded by *rv3790*), which synthesizes decaprenylphosphoryl- β -D- arabinose, and MoeW (encoded by *rv2338c*), an enzyme involved in the synthesis of a molybdenum-containing prosthetic group, the molybdenum cofactor. Under nutrient starvation conditions, overexpression of MoeW conferred partial resistance to TCA1 (102).

5.4.7 Nitro-containing compounds—A number of dual- and nonreplicating-active molecules contain a nitro functional group (Figure 11). The strongly electronegative nitro moiety has varying degrees of reactivity that depend on its local environment within a molecule. Mycobacterial nitroreductases may catalyze the conversion of the nitro group (-NO₂), by first reducing it to a nitroso (-NO), then to a hydroxylamine (-NHOH), and finally to an amine (-NH₂) (255, 256). Nitrofurans and nitroimidazoles undergo intrabacterial bioactivation to metabolites that can redox-cycle and cause oxidative damage, and/or dismutate to an electrophilic nitroso intermediate that reacts with intracellular thiols (257, 258). Reactive molecules such as nitrofurantoin may have specific, high affinity targets such as ribosomal proteins, yet become promiscuous at higher intrabacterial concentrations (258). The nitroso radical anion causes DNA oxidation and cell death due to DNA strand breaks (257). PA-824 donates a nitric oxide-like species that may have numerous intrabacterial targets (160, 180).

Examples of nitro-containing molecules that kill nonreplicating mycobacteria include metronidazole (19, 159, 161, 259); furaltadone (**compound 75**) (259); nitrofurantoin (**compound 76**) (96, 259); nitrofurazone (**compound 77**) (259); furazolidone (**compound 78**) (96); nitazoxanide (**compound 79**) (34, 50, 260); niclosamide (**compound 80**) (96); **compound 9** (113); nitrofuranylcalanolide (**compound 81**) (134); OPC-67683 (**compound**

82) (261, 262); PA-824 (50, 51, 96, 132, 152, 160, 263); TBA-354 (**compound 83**) (262); and **compound 84** (264).

While nitroimidazole-containing molecules such as PA-824 and OPC-67683 are in clinical trials, molecules containing the structurally similar nitrofuran moiety are considered hazardous due to the risk of DNA mutagenicity (265). Even if a molecule is non-toxic to eukaryotic cells, nitroimidazoles, nitrofurans, and nitrothiophenes should be assayed for mutagenicity (Table 4). The nitrofuranylcalanolide (**compound 81**) has potent MIC₉₀ values against replicating and nonreplicating *M. tuberculosis*, and kills *M. tuberculosis* infecting human macrophages (134). However, the nitrofuranylcalanolide was genotoxic *in vitro* (134).

Some nitro-containing molecules, such as nitazoxanide and niclosamide, are described in the “*Membrane depolarizers*” section.

5.4.8 Membrane depolarizers—Nonreplicating mycobacteria maintain an energized membrane (20). During hypoxia, depolarizing the mycobacterial membrane results in the inability to synthesize ATP by disrupting the intracellular to extracellular proton gradient (159, 166). Collapsing the proton motive force, and depleting intrabacterial ATP levels, may be a common feature of molecules that target replicating and nonreplicating mycobacteria (Figure 12) (266). The structure of a compound may not provide obvious clues that its anti-infective activity results from non-specific depolarization of the bacterial membrane. For example, some salicylanilides such as niclosamide shuttle protons by forming a stable 6-membered ring via hydrogen bonding (Figure 13) (267–269). The hydrogen-bonded form of salicylanilide has a higher logP than its non-hydrogen bonded structure, which is predicted to improve penetration across the lipophilic bacterial membrane. After passing into the bacterial cytosol and releasing its proton, the negatively charged salicylanilide anion, which is internally hydrogen bond stabilized, can return across the membrane again to pick up another proton (268).

Compounds that kill nonreplicating mycobacteria by depolarizing the membrane include the valinomycin (**compound 85**) (29, 159); nigericin (**compound 86**) (29, 132, 159); boromycin (**compound 87**) (270); nitazoxanide (34, 50, 260); monensin (**compound 88**) (120); salinomycin (**compound 89**) (132); niclosamide (96); a derivative of α -mangostin, A-0016 (**compound 90**) (271); N,N'-dicyclohexylcarbodiimide (DCCD, **compound 91**) (159); clofazimine (**compound 92**) (50, 55, 96, 272); and thioridazine (**compound 93**) (29, 51, 159, 161). The pro-drug clofazimine is reduced by type 2 NADH-quinone oxidoreductase. Clofazimine likely kills by interfering with mycobacterial respiration by generation of hydroxyl radicals (55, 273). The bactericidal action of clofazimine is reversed by addition of menaquinone-4 (MK-4) (273, 274). The phenothiazines thioridazine and trifluoperazine (**compound 94**) target the electron transport chain, disrupt membrane potential, and ultimately decrease ATP levels (275). Trifluoperazine was bactericidal to *M. tuberculosis* that was nonreplicating when cultured at acidic pH, in nutrient starved cultures, or at neutral pH in the presence of the nitric oxide donor DETA-NO (275). As described in the section on quinolones, TMC207 targets ATP synthase (AtpE, encoded by *rv1305*). **Compound 95**, which kills hypoxic *M. tuberculosis*, targets MenA (encoded by *rv0534c*) (110). Inhibition

of MenA, which catalyzes the prenylation of 1,4-dihydroxy-2-naphthoate to demethylmenaquinone, is anticipated to impact membrane potential and decrease ATP levels (110). N-octanesulphonylacetamide (OSA, **compound 96**) killed both replicating and hypoxic, nonreplicating *M. tuberculosis* (276–279). The rapid depletion in ATP levels after treating mycobacteria with **compound 97** was consistent with inhibition of the ATP synthase and/or the respiratory chain. The compound n-decanesulfonylacetamide (DSA, similar to **compound 96**) inhibits ATP synthase and lipid biosynthesis (276, 277, 279), killed hypoxic *M. bovis* BCG, and had bactericidal activity against class I rifampicin tolerant persisters (278). Q203 (**compound 97**) targets the cytochrome bc₁ complex (QcrB, encoded by *rv2196*), depletes ATP levels, kills replicating and hypoxic *M. tuberculosis*, and has activity in a mouse model of tuberculosis (280).

Dual active protonophores boast low frequencies of resistance. Membrane depolarization, and ensuing depletion of ATP, has an amplified downstream impact on the cell that cannot be easily compensated for by mutating a single gene. In the cases of nitazoxanide, boromycin, and AM-0016, the frequencies of resistance were estimated as $< 1 \times 10^{-12}$ (34), $< 1 \times 10^{-9}$ (270), and $< 1 \times 10^{-8}$ (271), respectively. Low frequencies of resistance are consistent with a non-specific mechanism of action or multiple specific mechanisms of action (281).

ATP depletion can lead to persister formation in *S. aureus* (282). It will be of interest to determine if clinical use of protonophores and membrane-targeting molecules selects for live, ATP-depleted, persister populations that are tolerant to other antibiotics.

5.4.9 More examples of molecules targeting nonreplicating mycobacteria—

Many compounds that are reported to kill nonreplicating mycobacteria do not fall into the categories described above (Figure 14). Auranofin (**compound 98**), a drug used to treat rheumatoid arthritis, was recently described as a potent inhibitor of mycobacterial thioredoxin reductase TrxB2 (encoded by *rv3913*) and had bactericidal activity against nutrient starved *M. tuberculosis* (283). While auranofin probably forms an Au(I) adduct with the cysteine active site of thioredoxin reductase, it has additional thiol targets (276, 283, 284). **Compound 99** inhibits CysM (encoded by *rv1336*), which has a critical role in mycobacterial cysteine biosynthesis, and killed nutrient-starved *M. tuberculosis* (107). DevR (also called DosR, encoded by *rv3133c*) controls expression of genes in response to hypoxia (109, 285). A DevR inhibitor, **compound 100**, killed *M. tuberculosis* that was cultured under hypoxic conditions, but spared the bacilli when they were nonreplicating during nutrient starvation (286). A 2-thiopyridine, **compound 101**, killed both replicating and nonreplicating hypoxic and nutrient-starved *M. tuberculosis* (287). The 2-thiopyridines were bactericidal to viable-but-not-culturable *M. tuberculosis* in a potassium starvation model (287). **Compound 102**, an inhibitor of pantothenate synthase (PanC, encoded by *rv3602c*) killed *M. tuberculosis* in a nutrient starvation model, and killed *M. marinum* infecting zebrafish (111). **Compound 103** killed hypoxic *M. tuberculosis* (288). A quinoxaline 1,4-di-N-oxide, **compound 104**, displayed potent activity against replicating and hypoxic nonreplicating *M. tuberculosis* and was active in an acute mouse model (289).

6. Future studies and conclusions

Phenotypic tolerance is complex. We must refrain from projecting the biology deciphered in one persistence model to other persistence models, for fear that we oversimplify phenotypic tolerance, much as Jacques Monod oversimplified biochemical unity when he declared, “*anything found to be true of E. coli must also be true of elephants*” (290). We need to focus time and resources on developing antibiotics that target mycobacteria in *in vitro* states that have relevance to persisters found during human tuberculosis, whose structures are accurately understood under the conditions of the assays, that exert *bona fide* bactericidal activity against nonreplicating mycobacteria, and that have a structure-activity relationship which enables at least one of its derivative compounds to make it through the gauntlet of drug development, or at least, to become an informative tool for chemical biology to guide our understanding of bacterial persistence (291, 292).

The challenges are formidable. Our confidence in *in vitro* screens is shaken by the dissociation between the high value of pyrazinamide in the clinic and its lack of activity *in vitro* at the highest concentrations typically considered appropriate for screening. We know many ways to put *M. tuberculosis* into a nonreplicating state *in vitro* but are not confident which states model the class II phenotypic tolerance that is hypothesized to contribute to the inefficiency of conventional drugs against tuberculosis in the human host. We need to validate compounds in animal models but are not sure which animal models (if any) qualitatively and quantitatively mimic which aspects of the human disease (42, 293). It is commonly noted that all current tuberculosis drugs work in mice, but compounds inactive in mouse models of tuberculosis have, to our knowledge, not been tested in primates or humans. A number of compounds active against replicating or nonreplicating *M. tuberculosis in vitro* and displaying suitable pharmacokinetics for studies in mice have failed to show efficacy in mice, but unless they were toxic, we almost never learn why they failed, or if the question is answered, the answer is almost never published. Such failures fail again when little or no insight is gained from the investment that led to the *in vivo* tests.

Two recent developments nonetheless justify optimism. First, academic microbiologists, immunologists, biochemists, structural biologists and physicians interested in these challenges are now working alongside professional medicinal chemists and pharmacologists. Second, these partnerships involve multiple academic institutions and multiple pharmaceutical companies, and the participants share their questions, hypotheses, failures and successes as they go along.

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References

1. Nathan C. Making space for anti-infective drug discovery. *Cell Host Microbe*. 2011; 9(5):343–348. PubMed PMID: 21575903. [PubMed: 21575903]
2. Davies J, Davies D. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev*. 2010; 74(3):417–433. PubMed PMID: 20805405; PubMed Central PMCID: PMC2937522. [PubMed: 20805405]
3. Hobby GL, Meyer K, Chaffee E. Observations on the Mechanism of Action of Penicillin. *Experimental Biology and Medicine*. 1942; 50(2):281–285.
4. Bigger J. Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *The Lancet*. 1944; 244(6320):497–500.
5. Hobby GL, Lenert TF. The in vitro action of antituberculous agents against multiplying and non-multiplying microbial cells. *American review of tuberculosis*. 1957; 76(6):1031–1048. PubMed PMID: 13488013. [PubMed: 13488013]
6. Koul A, Arnoult E, Lounis N, Guillemont J, Andries K. The challenge of new drug discovery for tuberculosis. *Nature*. 2011; 469(7331):483–490. PubMed PMID: 21270886. [PubMed: 21270886]
7. McCune RM, Feldmann FM, Lambert HP, McDermott W. Microbial persistence. I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. *J Exp Med*. 1966; 123(3):445–468. PubMed PMID: 4957010; PubMed Central PMCID: PMC2138153. [PubMed: 4957010]
8. Scanga CA, Mohan VP, Joseph H, Yu K, Chan J, Flynn JL. Reactivation of latent tuberculosis: variations on the Cornell murine model. *Infect Immun*. 1999; 67(9):4531–4538. Epub 1999/08/24. PubMed PMID: 10456896; PubMed Central PMCID: PMC96774. [PubMed: 10456896]
9. Pai SR, Actor JK, Sepulveda E, Hunter RL Jr, Jagannath C. Identification of viable and non-viable *Mycobacterium tuberculosis* in mouse organs by directed RT-PCR for antigen 85B mRNA. *Microbial pathogenesis*. 2000; 28(6):335–342. PubMed PMID: 10839970. [PubMed: 10839970]
10. Mukamolova GV, Turapov O, Malkin J, Woltmann G, Barer MR. Resuscitation-promoting factors reveal an occult population of tubercle Bacilli in Sputum. *Am J Respir Crit Care Med*. 2010; 181(2):174–180. PubMed PMID: 19875686; PubMed Central PMCID: PMC2809243. [PubMed: 19875686]
11. Chengalroyen MD, Beukes GM, Gordhan BG, Streicher EM, Churchyard G, Hafner R, et al. Detection and Quantification of Differentially Culturable Tubercle Bacteria in Sputum from Tuberculosis Patients. *Am J Respir Crit Care Med*. 2016 PubMed PMID: 27387272.
12. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol Microbiol*. 2002; 43(3):717–731. Epub 2002/04/04. PubMed PMID: 11929527. [PubMed: 11929527]
13. Brooks JV, Furney SK, Orme IM. Metronidazole therapy in mice infected with tuberculosis. *Antimicrob Agents Chemother*. 1999; 43(5):1285–1288. PubMed PMID: 10223954; PubMed Central PMCID: PMC89261. [PubMed: 10223954]
14. Carroll MW, Jeon D, Mountz JM, Lee JD, Jeong YJ, Zia N, et al. Efficacy and safety of metronidazole for pulmonary multidrug-resistant tuberculosis. *Antimicrob Agents Chemother*. 2013; 57(8):3903–3909. PubMed PMID: 23733467; PubMed Central PMCID: PMC3719751. [PubMed: 23733467]
15. Hoff DR, Caraway ML, Brooks EJ, Driver ER, Ryan GJ, Peloquin CA, et al. Metronidazole lacks antibacterial activity in guinea pigs infected with *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2008; 52(11):4137–4140. PubMed PMID: 18694944; PubMed Central PMCID: PMC2573095. [PubMed: 18694944]
16. Lin PL, Dartois V, Johnston PJ, Janssen C, Via L, Goodwin MB, et al. Metronidazole prevents reactivation of latent *Mycobacterium tuberculosis* infection in macaques. *Proc Natl Acad Sci U S A*. 2012; 109(35):14188–14193. PubMed PMID: 22826237; PubMed Central PMCID: PMC3435210. [PubMed: 22826237]

17. Via LE, Lin PL, Ray SM, Carrillo J, Allen SS, Eum SY, et al. Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infect Immun*. 2008; 76(6):2333–2340. PubMed PMID: 18347040; PubMed Central PMCID: PMC2423064. [PubMed: 18347040]
18. Wayne LG. Dormancy of *Mycobacterium tuberculosis* and latency of disease. *Eur J Clin Microbiol Infect Dis*. 1994; 13(11):908–914. Epub 1994/11/01. PubMed PMID: 7698116. [PubMed: 7698116]
19. Wayne LG, Sramek HA. Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 1994; 38(9):2054–2058. PubMed PMID: 7811018; PubMed Central PMCID: PMC284683. [PubMed: 7811018]
20. Boshoff HI, Barry CE 3rd. Tuberculosis - metabolism and respiration in the absence of growth. *Nat Rev Microbiol*. 2005; 3(1):70–80. PubMed PMID: 15608701. [PubMed: 15608701]
21. Cunningham-Bussell A, Zhang T, Nathan CF. Nitrite produced by *Mycobacterium tuberculosis* in human macrophages in physiologic oxygen impacts bacterial ATP consumption and gene expression. *Proc Natl Acad Sci U S A*. 2013; 110(45):E4256–E4265. Epub 2013/10/23. PubMed PMID: 24145454; PubMed Central PMCID: PMC3831502. [PubMed: 24145454]
22. Watanabe S, Zimmermann M, Goodwin MB, Sauer U, Barry CE 3rd, Boshoff HI. Fumarate reductase activity maintains an energized membrane in anaerobic *Mycobacterium tuberculosis*. *PLoS pathogens*. 2011; 7(10):e1002287. PubMed PMID: 21998585; PubMed Central PMCID: PMC3188519. [PubMed: 21998585]
23. Wade MM, Zhang Y. Anaerobic incubation conditions enhance pyrazinamide activity against *Mycobacterium tuberculosis*. *J Med Microbiol*. 2004; 53(Pt 8):769–773. PubMed PMID: 15272064. [PubMed: 15272064]
24. Coates A, Hu Y, Bax R, Page C. The future challenges facing the development of new antimicrobial drugs. *Nature reviews Drug discovery*. 2002; 1(11):895–910. Epub 2002/11/05. PubMed PMID: 12415249. [PubMed: 12415249]
25. Nathan C. Fresh approaches to anti-infective therapies. *Science translational medicine*. 2012; 4(140):140sr2. PubMed PMID: 22745440. [PubMed: 22745440]
26. Nathan C. Cooperative development of antimicrobials: looking back to look ahead. *Nat Rev Micro*. 2015; 13(10):651–657.
27. Nathan C, Barry CE 3rd. TB drug development: immunology at the table. *Immunol Rev*. 2015; 264(1):308–318. PubMed PMID: 25703568; PubMed Central PMCID: PMC4339218. [PubMed: 25703568]
28. Warriar T, Martinez-Hoyos M, Marin-Amieva M, Colmenarejo G, Porras-De Francisco E, Alvarez-Pedraglio AI, et al. Identification of Novel Anti-mycobacterial Compounds by Screening a Pharmaceutical Small-Molecule Library against Nonreplicating *Mycobacterium tuberculosis*. *ACS Infectious Diseases*. 2015
29. Mak PA, Rao SP, Ping Tan M, Lin X, Chyba J, Tay J, et al. A high-throughput screen to identify inhibitors of ATP homeostasis in non-replicating *Mycobacterium tuberculosis*. *ACS chemical biology*. 2012; 7(7):1190–1197. PubMed PMID: 22500615; PubMed Central PMCID: PMC3401038. [PubMed: 22500615]
30. Gold B, Roberts J, Ling Y, Lopez Quezada L, Glasheen J, Ballinger E, et al. Rapid, semi-quantitative assay to discriminate among compounds with activity against replicating or non-replicating *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2015 PubMed PMID: 26239979.
31. Grosset JH, Tyagi S, Almeida DV, Converse PJ, Li SY, Ammerman NC, et al. Assessment of clofazimine activity in a second-line regimen for tuberculosis in mice. *Am J Respir Crit Care Med*. 2013; 188(5):608–612. PubMed PMID: 23822735; PubMed Central PMCID: PMC3827279. [PubMed: 23822735]
32. Lounis N, Gevers T, Van Den Berg J, Verhaeghe T, van Heeswijk R, Andries K. Prevention of drug carryover effects in studies assessing antimycobacterial efficacy of TMC207. *J Clin Microbiol*. 2008; 46(7):2212–2215. PubMed PMID: 18480227. [PubMed: 18480227]
33. Tasneen R, Williams K, Amoabeng O, Minkowski A, Mdluli KE, Upton AM, et al. Contribution of the nitroimidazoles PA-824 and TBA-354 to the activity of novel regimens in murine models of

- tuberculosis. *Antimicrob Agents Chemother.* 2015; 59(1):129–135. PubMed PMID: 25331697; PubMed Central PMCID: PMC4291340. [PubMed: 25331697]
34. de Carvalho LP, Lin G, Jiang X, Nathan C. Nitazoxanide kills replicating and nonreplicating *Mycobacterium tuberculosis* and evades resistance. *J Med Chem.* 2009; 52(19):5789–5792. PubMed PMID: 19736929. [PubMed: 19736929]
 35. Roostalu J, Joers A, Luidalepp H, Kaldalu N, Tenson T. Cell division in *Escherichia coli* cultures monitored at single cell resolution. *BMC microbiology.* 2008; 8:68. PubMed PMID: 18430255; PubMed Central PMCID: PMC2377270. [PubMed: 18430255]
 36. Orman MA, Brynildsen MP. Dormancy is not necessary or sufficient for bacterial persistence. *Antimicrob Agents Chemother.* 2013; 57(7):3230–3239. PubMed PMID: 23629720; PubMed Central PMCID: PMC3697331. [PubMed: 23629720]
 37. Vega NM, Allison KR, Khalil AS, Collins JJ. Signaling-mediated bacterial persister formation. *Nat Chem Biol.* 2012; 8(5):431–433. PubMed PMID: 22426114; PubMed Central PMCID: PMC3329571. [PubMed: 22426114]
 38. Dhar N, Dubee V, Ballell L, Cuinet G, Hugonnet JE, Signorino-Gelo F, et al. Rapid cytolysis of *Mycobacterium tuberculosis* by faropenem, an orally bioavailable beta-lactam antibiotic. *Antimicrob Agents Chemother.* 2015; 59(2):1308–1319. PubMed PMID: 25421469; PubMed Central PMCID: PMC4335862. [PubMed: 25421469]
 39. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. *Science.* 2004; 305(5690):1622–1625. PubMed PMID: 15308767. [PubMed: 15308767]
 40. Allison KR, Brynildsen MP, Collins JJ. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature.* 2011; 473(7346):216–220. PubMed PMID: 21562562; PubMed Central PMCID: PMC3145328. [PubMed: 21562562]
 41. Allison KR, Brynildsen MP, Collins JJ. Heterogeneous bacterial persisters and engineering approaches to eliminate them. *Curr Opin Microbiol.* 2011; 14(5):593–598. PubMed PMID: 21937262; PubMed Central PMCID: PMC3196368. [PubMed: 21937262]
 42. Prideaux B, Via LE, Zimmerman MD, Eum S, Sarathy J, O'Brien P, et al. The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nat Med.* 2015; 21(10):1223–1227. PubMed PMID: 26343800; PubMed Central PMCID: PMC4598290. [PubMed: 26343800]
 43. Muttucumaru DG, Roberts G, Hinds J, Stabler RA, Parish T. Gene expression profile of *Mycobacterium tuberculosis* in a non-replicating state. *Tuberculosis (Edinb).* 2004; 84(3-4):239–246. PubMed PMID: 15207493. [PubMed: 15207493]
 44. Voskuil MI, Visconti KC, Schoolnik GK. *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis (Edinb).* 2004; 84(3-4):218–227. PubMed PMID: 15207491. [PubMed: 15207491]
 45. Talaat AM, Howard ST, Hale Wt, Lyons R, Garner H, Johnston SA. Genomic DNA standards for gene expression profiling in *Mycobacterium tuberculosis*. *Nucleic Acids Res.* 2002; 30(20):e104. PubMed PMID: 12384606; PubMed Central PMCID: PMC137148. [PubMed: 12384606]
 46. Keren I, Minami S, Rubin E, Lewis K. Characterization and transcriptome analysis of *Mycobacterium tuberculosis* persisters. *mBio.* 2011; 2(3):e00100–e00111. Epub 2011/06/16. PubMed PMID: 21673191; PubMed Central PMCID: PMC3119538. [PubMed: 21673191]
 47. Benjak A, Uplekar S, Zhang M, Piton J, Cole ST, Sala C. Genomic and transcriptomic analysis of the streptomycin-dependent *Mycobacterium tuberculosis* strain 18b. *BMC genomics.* 2016; 17(1):190. PubMed PMID: 26944551; PubMed Central PMCID: PMC4779234. [PubMed: 26944551]
 48. Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, et al. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med.* 2003; 198(5):705–713. PubMed PMID: 12953092. [PubMed: 12953092]
 49. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, et al. Transcriptional Adaptation of *Mycobacterium tuberculosis* within Macrophages: Insights into the Phagosomal Environment. *J Exp Med.* 2003; 198(5):693–704. PubMed PMID: 12953091; PubMed Central PMCID: PMC2194186. [PubMed: 12953091]
 50. Franzblau SG, DeGroot MA, Cho SH, Andries K, Nuermberger E, Orme IM, et al. Comprehensive analysis of methods used for the evaluation of compounds against *Mycobacterium*

tuberculosis. Tuberculosis (Edinb). 2012; 92(6):453–488. PubMed PMID: 22940006. [PubMed: 22940006]

51. Lakshminarayana SB, Huat TB, Ho PC, Manjunatha UH, Dartois V, Dick T, et al. Comprehensive physicochemical, pharmacokinetic and activity profiling of anti-TB agents. J Antimicrob Chemother. 2015; 70(3):857–867. PubMed PMID: 25587994. [PubMed: 25587994]
52. Xie Z, Siddiqi N, Rubin EJ. Differential antibiotic susceptibilities of starved *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother. 2005; 49(11):4778–4780. PubMed PMID: 16251329; PubMed Central PMCID: PMC1280169. [PubMed: 16251329]
53. Gold B, Pingle M, Brickner SJ, Shah N, Roberts J, Rundell M, et al. Nonsteroidal anti-inflammatory drug sensitizes *Mycobacterium tuberculosis* to endogenous and exogenous antimicrobials. Proc Natl Acad Sci U S A. 2012; 109(40):16004–16011. PubMed PMID: 23012453; PubMed Central PMCID: PMC3479555. [PubMed: 23012453]
54. Grant SS, Kawate T, Nag PP, Silvis MR, Gordon K, Stanley SA, et al. Identification of novel inhibitors of nonreplicating *Mycobacterium tuberculosis* using a carbon starvation model. ACS chemical biology. 2013; 8(10):2224–2234. PubMed PMID: 23898841; PubMed Central PMCID: PMC3864639. [PubMed: 23898841]
55. Grant SS, Kaufmann BB, Chand NS, Haseley N, Hung DT. Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals. Proc Natl Acad Sci U S A. 2012; 109(30):12147–12152. PubMed PMID: 22778419; PubMed Central PMCID: PMC3409745. [PubMed: 22778419]
56. Tuomanen E. Phenotypic tolerance: the search for beta-lactam antibiotics that kill nongrowing bacteria. Reviews of infectious diseases. 1986; 8(Suppl 3):S279–S291. PubMed PMID: 3529321. [PubMed: 3529321]
57. Torrey HL, Keren I, Via LE, Lee JS, Lewis K. High Persister Mutants in *Mycobacterium tuberculosis*. PLoS One. 2016; 11(5):e0155127. PubMed PMID: 27176494; PubMed Central PMCID: PMC4866775. [PubMed: 27176494]
58. Pattyn SR, Dockx P, Rollier MT, Rollier R, Saerens EJ. *Mycobacterium leprae* persisters after treatment with dapsone and rifampicin. Int J Lepr Other Mycobact Dis. 1976; 44(1-2):154–158. PubMed PMID: 776851. [PubMed: 776851]
59. Mulcahy LR, Burns JL, Lory S, Lewis K. Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol. 2010; 192(23):6191–6199. PubMed PMID: 20935098; PubMed Central PMCID: PMC2981199. [PubMed: 20935098]
60. Lafleur MD, Qi Q, Lewis K. Patients with long-term oral carriage harbor high-persister mutants of *Candida albicans*. Antimicrob Agents Chemother. 2010; 54(1):39–44. PubMed PMID: 19841146; PubMed Central PMCID: PMC2798516. [PubMed: 19841146]
61. Schumacher MA, Balani P, Min J, Chinnam NB, Hansen S, Vulic M, et al. HipBA-promoter structures reveal the basis of heritable multidrug tolerance. Nature. 2015; 524(7563):59–64. PubMed PMID: 26222023. [PubMed: 26222023]
62. Ahmad Z, Klinkenberg LG, Pinn ML, Fraig MM, Peloquin CA, Bishai WR, et al. Biphasic kill curve of isoniazid reveals the presence of drug-tolerant, not drug-resistant *Mycobacterium tuberculosis* in the guinea pig. J Infect Dis. 2009; 200(7):1136–1143. PubMed PMID: 19686043. [PubMed: 19686043]
63. Ahmad Z, Pinn ML, Nuermberger EL, Peloquin CA, Grosset JH, Karakousis PC. The potent bactericidal activity of streptomycin in the guinea pig model of tuberculosis ceases due to the presence of persisters. J Antimicrob Chemother. 2010; 65(10):2172–2175. PubMed PMID: 20693172; PubMed Central PMCID: PMC2941674. [PubMed: 20693172]
64. Driver ER, Ryan GJ, Hoff DR, Irwin SM, Basaraba RJ, Kramnik I, et al. Evaluation of a mouse model of necrotic granuloma formation using C3HeB/FeJ mice for testing of drugs against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2012; 56(6):3181–3195. PubMed PMID: 22470120; PubMed Central PMCID: PMC3370740. [PubMed: 22470120]
65. Singh R, Barry CE 3rd, Boshoff HI. The three RelE homologs of *Mycobacterium tuberculosis* have individual, drug-specific effects on bacterial antibiotic tolerance. J Bacteriol. 2010; 192(5):1279–1291. PubMed PMID: 20061486; PubMed Central PMCID: PMC2820853. [PubMed: 20061486]

66. Nandakumar M, Nathan C, Rhee KY. Isocitrate lyase mediates broad antibiotic tolerance in *Mycobacterium tuberculosis*. *Nature communications*. 2014; 5:4306. PubMed PMID: 24978671.
67. Wiuff C, Zappala RM, Regoes RR, Garner KN, Baquero F, Levin BR. Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. *Antimicrob Agents Chemother*. 2005; 49(4):1483–1494. PubMed PMID: 15793130; PubMed Central PMCID: PMC1068602. [PubMed: 15793130]
68. Kim JS, Heo P, Yang TJ, Lee KS, Cho DH, Kim BT, et al. Selective killing of bacterial persisters by a single chemical compound without affecting normal antibiotic-sensitive cells. *Antimicrob Agents Chemother*. 2011; 55(11):5380–5383. PubMed PMID: 21844322; PubMed Central PMCID: PMC3195057. [PubMed: 21844322]
69. Black DS, Irwin B, Moyed HS. Autoregulation of hip, an operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis. *J Bacteriol*. 1994; 176(13):4081–4091. PubMed PMID: 8021189; PubMed Central PMCID: PMC205607. [PubMed: 8021189]
70. Black DS, Kelly AJ, Mardis MJ, Moyed HS. Structure and organization of hip, an operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis. *J Bacteriol*. 1991; 173(18):5732–5739. PubMed PMID: 1715862; PubMed Central PMCID: PMC208304. [PubMed: 1715862]
71. Moyed HS, Bertrand KP. hipA, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J Bacteriol*. 1983; 155(2):768–775. PubMed PMID: 6348026; PubMed Central PMCID: PMC217749. [PubMed: 6348026]
72. Moyed HS, Broderick SH. Molecular cloning and expression of hipA, a gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J Bacteriol*. 1986; 166(2):399–403. PubMed PMID: 3516974; PubMed Central PMCID: PMC214618. [PubMed: 3516974]
73. Slattery A, Victorsen AH, Brown A, Hillman K, Phillips GJ. Isolation of highly persistent mutants of *Salmonella enterica* serovar typhimurium reveals a new toxin-antitoxin module. *J Bacteriol*. 2013; 195(4):647–657. PubMed PMID: 23204462; PubMed Central PMCID: PMC3562109. [PubMed: 23204462]
74. Maisonneuve E, Gerdes K. Molecular mechanisms underlying bacterial persisters. *Cell*. 2014; 157(3):539–548. PubMed PMID: 24766804. [PubMed: 24766804]
75. Lewis K. Persister cells: molecular mechanisms related to antibiotic tolerance. *Handbook of experimental pharmacology*. 2012; (211):121–133. PubMed PMID: 23090599.
76. Lewis K. Persister cells. *Annu Rev Microbiol*. 2010; 64:357–372. PubMed PMID: 20528688. [PubMed: 20528688]
77. Lewis K. Multidrug tolerance of biofilms and persister cells. *Current topics in microbiology and immunology*. 2008; 322:107–131. PubMed PMID: 18453274. [PubMed: 18453274]
78. Lewis K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol*. 2007; 5(1):48–56. PubMed PMID: 17143318. [PubMed: 17143318]
79. Conlon BP, Rowe SE, Lewis K. Persister cells in biofilm associated infections. *Advances in experimental medicine and biology*. 2015; 831:1–9. PubMed PMID: 25384659. [PubMed: 25384659]
80. Aldridge BB, Fernandez-Suarez M, Heller D, Ambravaneswaran V, Irimia D, Toner M, et al. Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science*. 2012; 335(6064):100–104. PubMed PMID: 22174129; PubMed Central PMCID: PMC3397429. [PubMed: 22174129]
81. Vaubourgeix J, Lin G, Dhar N, Chenouard N, Jiang X, Botella H, et al. Stressed mycobacteria use the chaperone ClpB to sequester irreversibly oxidized proteins asymmetrically within and between cells. *Cell Host Microbe*. 2015; 17(2):178–190. PubMed PMID: 25620549. [PubMed: 25620549]
82. Keren I, Shah D, Spoering A, Kaldalu N, Lewis K. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bacteriol*. 2004; 186(24):8172–8180. PubMed PMID: 15576765; PubMed Central PMCID: PMC532439. [PubMed: 15576765]
83. Maisonneuve E, Shakespeare LJ, Jorgensen MG, Gerdes K. Bacterial persistence by RNA endonucleases. *Proc Natl Acad Sci U S A*. 2011; 108(32):13206–13211. PubMed PMID: 21788497; PubMed Central PMCID: PMC3156201. [PubMed: 21788497]

84. Sala A, Bordes P, Genevax P. Multiple toxin-antitoxin systems in *Mycobacterium tuberculosis*. *Toxins*. 2014; 6(3):1002–1020. PubMed PMID: 24662523; PubMed Central PMCID: PMC3968373. [PubMed: 24662523]
85. Javid B, Sorrentino F, Toosky M, Zheng W, Pinkham JT, Jain N, et al. Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance. *Proc Natl Acad Sci U S A*. 2014; 111(3):1132–1137. PubMed PMID: 24395793; PubMed Central PMCID: PMC3903211. [PubMed: 24395793]
86. Su HW, Zhu JH, Li H, Cai RJ, Ealand C, Wang X, et al. The essential mycobacterial amidotransferase GatCAB is a modulator of specific translational fidelity. *Nature microbiology*. 2016; 1:16147. PubMed PMID: 27564922.
87. Dhar N, McKinney JD. *Mycobacterium tuberculosis* persistence mutants identified by screening in isoniazid-treated mice. *Proc Natl Acad Sci U S A*. 2010; 107(27):12275–12280. PubMed PMID: 20566858; PubMed Central PMCID: PMC2901468. [PubMed: 20566858]
88. Raj A, Peskin CS, Tranchina D, Vargas DY, Tyagi S. Stochastic mRNA synthesis in mammalian cells. *PLoS biology*. 2006; 4(10):e309. PubMed PMID: 17048983; PubMed Central PMCID: PMC1563489. [PubMed: 17048983]
89. Maamar H, Raj A, Dubnau D. Noise in gene expression determines cell fate in *Bacillus subtilis*. *Science*. 2007; 317(5837):526–529. PubMed PMID: 17569828; PubMed Central PMCID: PMC3828679. [PubMed: 17569828]
90. Wakamoto Y, Dhar N, Chait R, Schneider K, Signorino-Gelo F, Leibler S, et al. Dynamic persistence of antibiotic-stressed mycobacteria. *Science*. 2013; 339(6115):91–95. PubMed PMID: 23288538. [PubMed: 23288538]
91. Debbia EA, Roveta S, Schito AM, Gualco L, Marchese A. Antibiotic persistence: the role of spontaneous DNA repair response. *Microbial drug resistance*. 2001; 7(4):335–342. PubMed PMID: 11822773. [PubMed: 11822773]
92. Theodore A, Lewis K, Vulic M. Tolerance of *Escherichia coli* to fluoroquinolone antibiotics depends on specific components of the SOS response pathway. *Genetics*. 2013; 195(4):1265–1276. PubMed PMID: 24077306; PubMed Central PMCID: PMC3832272. [PubMed: 24077306]
93. Dorr T, Lewis K, Vulic M. SOS response induces persistence to fluoroquinolones in *Escherichia coli*. *PLoS genetics*. 2009; 5(12):e1000760. PubMed PMID: 20011100; PubMed Central PMCID: PMC2780357. [PubMed: 20011100]
94. Gold, B., Warriar, T., Nathan, C. A Multi-Stress Model for High Throughput Screening Against Non-replicating *Mycobacterium tuberculosis*. In: Parish, T., Roberts, D., editors. *Mycobacteria Protocols, Methods in Molecular Biology*. 1285. Third. Springer; 2015. p. 293-315.
95. Wayne LG, Hayes LG. An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun*. 1996; 64(6):2062–2069. PubMed PMID: 8675308; PubMed Central PMCID: PMC174037. [PubMed: 8675308]
96. Cho SH, Warit S, Wan B, Hwang CH, Pauli GF, Franzblau SG. Low-oxygen-recovery assay for high-throughput screening of compounds against nonreplicating *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2007; 51(4):1380–1385. Epub 2007/01/11. PubMed PMID: 17210775; PubMed Central PMCID: PMC1855511. [PubMed: 17210775]
97. Schnappinger D, Schoolnik GK, Ehrt S. Expression profiling of host pathogen interactions: how *Mycobacterium tuberculosis* and the macrophage adapt to one another. *Microbes Infect*. 2006; 8(4):1132–1140. Epub 2006/03/07. PubMed PMID: 16517202. [PubMed: 16517202]
98. Lavollay M, Arthur M, Fourgeaud M, Dubost L, Marie A, Veziris N, et al. The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. *J Bacteriol*. 2008; 190(12):4360–4366. PubMed PMID: 18408028; PubMed Central PMCID: PMC2446752. [PubMed: 18408028]
99. Kumar P, Arora K, Lloyd JR, Lee IY, Nair V, Fischer E, et al. Meropenem inhibits D,D-carboxypeptidase activity in *Mycobacterium tuberculosis*. *Mol Microbiol*. 2012; 86(2):367–381. PubMed PMID: 22906310; PubMed Central PMCID: PMC3468717. [PubMed: 22906310]
100. Bryk R, Gold B, Venugopal A, Singh J, Samy R, Pupek K, et al. Selective killing of nonreplicating mycobacteria. *Cell Host Microbe*. 2008; 3(3):137–145. PubMed PMID: 18329613; PubMed Central PMCID: PMC2423947. [PubMed: 18329613]

101. Darby CM, Nathan CF. Killing of non-replicating *Mycobacterium tuberculosis* by 8-hydroxyquinoline. J Antimicrob Chemother. 2010; 65(7):1424–1427. PubMed PMID: 20435781. [PubMed: 20435781]
102. Wang F, Sambandan D, Halder R, Wang J, Batt SM, Weinrick B, et al. Identification of a small molecule with activity against drug-resistant and persistent tuberculosis. Proc Natl Acad Sci U S A. 2013; 110(27):E2510–E2517. PubMed PMID: 23776209; PubMed Central PMCID: PMC3703973. [PubMed: 23776209]
103. Zhang M, Sala C, Dhar N, Vocat A, Sambandamurthy VK, Sharma S, et al. In vitro and in vivo activities of three oxazolidinones against nonreplicating *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2014; 58(6):3217–3223. PubMed PMID: 24663022; PubMed Central PMCID: PMC4068502. [PubMed: 24663022]
104. Zhang M, Sala C, Hartkoorn RC, Dhar N, Mendoza-Losana A, Cole ST. Streptomycin-starved *Mycobacterium tuberculosis* 18b, a drug discovery tool for latent tuberculosis. Antimicrob Agents Chemother. 2012; 56(11):5782–5789. PubMed PMID: 22926567; PubMed Central PMCID: PMC3486556. [PubMed: 22926567]
105. Bassett IM, Lun S, Bishai WR, Guo H, Kirman JR, Altaf M, et al. Detection of inhibitors of phenotypically drug-tolerant *Mycobacterium tuberculosis* using an in vitro bactericidal screen. Journal of microbiology. 2013; 51(5):651–658. PubMed PMID: 23800952.
106. Lin G, Li D, de Carvalho LP, Deng H, Tao H, Vogt G, et al. Inhibitors selective for mycobacterial versus human proteasomes. Nature. 2009; 461(7264):621–626. Epub 2009/09/18. PubMed PMID: 19759536. [PubMed: 19759536]
107. Brunner K, Maric S, Reshma RS, Almqvist H, Seashore-Ludlow B, Gustavsson AL, et al. Inhibitors of the Cysteine Synthase CysM with Antibacterial Potency against Dormant *Mycobacterium tuberculosis*. J Med Chem. 2016 PubMed PMID: 27379713.
108. Chopra S, Matsuyama K, Tran T, Malerich JP, Wan B, Franzblau SG, et al. Evaluation of gyrase B as a drug target in *Mycobacterium tuberculosis*. J Antimicrob Chemother. 2012; 67(2):415–421. PubMed PMID: 22052686; PubMed Central PMCID: PMC3254195. [PubMed: 22052686]
109. Dasgupta N, Kapur V, Singh KK, Das TK, Sachdeva S, Jyothisri K, et al. Characterization of a two-component system, devR-devS, of *Mycobacterium tuberculosis*. Tuber Lung Dis. 2000; 80(3):141–159. PubMed PMID: 10970762. [PubMed: 10970762]
110. Debnath J, Siricilla S, Wan B, Crick DC, Lenaerts AJ, Franzblau SG, et al. Discovery of selective menaquinone biosynthesis inhibitors against *Mycobacterium tuberculosis*. J Med Chem. 2012; 55(8):3739–3755. PubMed PMID: 22449052; PubMed Central PMCID: PMC3375340. [PubMed: 22449052]
111. Samala G, Devi PB, Saxena S, Meda N, Yogeewari P, Sriram D. Design, synthesis and biological evaluation of imidazo[2,1-b]thiazole and benzo[d]imidazo[2,1-b]thiazole derivatives as *Mycobacterium tuberculosis* pantothenate synthetase inhibitors. Bioorg Med Chem. 2016; 24(6):1298–1307. PubMed PMID: 26867485. [PubMed: 26867485]
112. Shirude PS, Madhavapeddi P, Tucker JA, Murugan K, Patil V, Basavarajappa H, et al. Aminopyrazinamides: novel and specific GyrB inhibitors that kill replicating and nonreplicating *Mycobacterium tuberculosis*. ACS chemical biology. 2013; 8(3):519–523. PubMed PMID: 23268609. [PubMed: 23268609]
113. Sridevi JP, Suryadevara P, Janupally R, Sridhar J, Soni V, Anantaramu HS, et al. Identification of potential *Mycobacterium tuberculosis* topoisomerase I inhibitors: a study against active, dormant and resistant tuberculosis. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences. 2015; 72:81–92. PubMed PMID: 25769524. [PubMed: 25769524]
114. Olaleye O, Raghunand TR, Bhat S, Chong C, Gu P, Zhou J, et al. Characterization of clioquinol and analogues as novel inhibitors of methionine aminopeptidases from *Mycobacterium tuberculosis*. Tuberculosis (Edinb). 2011; 91(Suppl 1):S61–S95. Epub 2011/11/26. PubMed PMID: 22115541. [PubMed: 22115541]
115. Olaleye O, Raghunand TR, Bhat S, He J, Tyagi S, Lamichhane G, et al. Methionine aminopeptidases from *Mycobacterium tuberculosis* as novel antimycobacterial targets. Chem Biol. 2010; 17(1):86–97. PubMed PMID: 20142044; PubMed Central PMCID: PMC3165048. [PubMed: 20142044]

116. Chakraborty S, Gruber T, Barry CE 3rd, Boshoff HI, Rhee KY. Para-aminosalicylic acid acts as an alternative substrate of folate metabolism in *Mycobacterium tuberculosis*. *Science*. 2013; 339(6115):88–91. PubMed PMID: 23118010; PubMed Central PMCID: PMC3792487. [PubMed: 23118010]
117. Chakraborty S, Rhee KY. Tuberculosis Drug Development: History and Evolution of the Mechanism-Based Paradigm. *Cold Spring Harbor perspectives in medicine*. 2015; 5(8): a021147. PubMed PMID: 25877396. [PubMed: 25877396]
118. Vocat A, Hartkoorn RC, Lechartier B, Zhang M, Dhar N, Cole ST, et al. Bioluminescence for assessing drug potency against nonreplicating *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2015; 59(7):4012–4019. PubMed PMID: 25896710; PubMed Central PMCID: PMC4468706. [PubMed: 25896710]
119. Darby CM, Ingolfsson HI, Jiang X, Shen C, Sun M, Zhao N, et al. Whole cell screen for inhibitors of pH homeostasis in *Mycobacterium tuberculosis*. *PLoS One*. 2013; 8(7):e68942. PubMed PMID: 23935911; PubMed Central PMCID: PMC3728290. [PubMed: 23935911]
120. Darby CM, Ingolfsson HI, Jiang X, Shen C, Sun M, Zhao N, et al. Whole cell screen for inhibitors of pH homeostasis in *Mycobacterium tuberculosis*. *PLoS One*. 2013; 8(7):e68942. PubMed PMID: 23935911; PubMed Central PMCID: PMC3728290. [PubMed: 23935911]
121. Brook I. Inoculum effect. *Reviews of infectious diseases*. 1989; 11(3):361–368. PubMed PMID: 2664999. [PubMed: 2664999]
122. Dahl JL, Kraus CN, Boshoff HI, Doan B, Foley K, Avarbock D, et al. The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. *Proc Natl Acad Sci U S A*. 2003; 100(17):10026–10031. PubMed PMID: 12897239; PubMed Central PMCID: PMC187750. [PubMed: 12897239]
123. Zhao N, Darby CM, Small J, Bachovchin DA, Jiang X, Burns-Huang KE, et al. Target-based screen against a periplasmic serine protease that regulates intrabacterial pH homeostasis in *Mycobacterium tuberculosis*. *ACS chemical biology*. 2015; 10(2):364–371. PubMed PMID: 25457457; PubMed Central PMCID: PMC4340348. [PubMed: 25457457]
124. Vandal OH, Nathan CF, Ehrt S. Acid resistance in *Mycobacterium tuberculosis*. *J Bacteriol*. 2009; 191(15):4714–4721. Epub 2009/05/26. PubMed PMID: 19465648; PubMed Central PMCID: PMC2715723. [PubMed: 19465648]
125. Vandal OH, Pierini LM, Schnappinger D, Nathan CF, Ehrt S. A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. *Nat Med*. 2008; 14(8):849–854. Epub 2008/07/22. PubMed PMID: 18641659; PubMed Central PMCID: PMC2538620. [PubMed: 18641659]
126. Vandal OH, Roberts JA, Odaira T, Schnappinger D, Nathan CF, Ehrt S. Acid-susceptible mutants of *Mycobacterium tuberculosis* share hypersusceptibility to cell wall and oxidative stress and to the host environment. *J Bacteriol*. 2009; 191(2):625–631. Epub 2008/11/18. PubMed PMID: 19011036; PubMed Central PMCID: PMC2620805. [PubMed: 19011036]
127. Miesenbock G, De Angelis DA, Rothman JE. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*. 1998; 394(6689):192–195. PubMed PMID: 9671304. [PubMed: 9671304]
128. Ackart DF, Hascall-Dove L, Caceres SM, Kirk NM, Podell BK, Melander C, et al. Expression of antimicrobial drug tolerance by attached communities of *Mycobacterium tuberculosis*. *Pathogens and disease*. 2014; 70(3):359–369. PubMed PMID: 24478060; PubMed Central PMCID: PMC4361083. [PubMed: 24478060]
129. Recht J, Kolter R. Glycopeptidolipid acetylation affects sliding motility and biofilm formation in *Mycobacterium smegmatis*. *J Bacteriol*. 2001; 183(19):5718–5724. PubMed PMID: 11544235; PubMed Central PMCID: PMC95464. [PubMed: 11544235]
130. Recht J, Martinez A, Torello S, Kolter R. Genetic analysis of sliding motility in *Mycobacterium smegmatis*. *J Bacteriol*. 2000; 182(15):4348–4351. PubMed PMID: 10894747; PubMed Central PMCID: PMC101957. [PubMed: 10894747]
131. Piccaro G, Giannoni F, Filippini P, Mustazzolu A, Fattorini L. Activities of drug combinations against *Mycobacterium tuberculosis* grown in aerobic and hypoxic acidic conditions. *Antimicrob Agents Chemother*. 2013; 57(3):1428–1433. PubMed PMID: 23295931; PubMed Central PMCID: PMC3591868. [PubMed: 23295931]

132. Sala C, Dhar N, Hartkoorn RC, Zhang M, Ha YH, Schneider P, et al. Simple model for testing drugs against nonreplicating *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2010; 54(10):4150–4158. PubMed PMID: 20679505; PubMed Central PMCID: PMC2944619. [PubMed: 20679505]
133. Hartkoorn RC, Ryabova OB, Chiarelli LR, Riccardi G, Makarov V, Cole ST. Mechanism of action of 5-nitrothiophenes against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2014; 58(5):2944–2947. PubMed PMID: 24550336; PubMed Central PMCID: PMC3993272. [PubMed: 24550336]
134. Zheng P, Somersan-Karakaya S, Lu S, Roberts J, Pingle M, Warriar T, et al. Synthetic Calanolides with Bactericidal Activity Against Replicating and Nonreplicating *Mycobacterium tuberculosis*. *J Med Chem*. 2014 PubMed PMID: 24694175.
135. Warriar T, Martinez-Hoyos M, Marin-Amieva M, Colmenarejo G, Porras E, Alvarez-Pedraglio AI, et al. Identification of novel anti-mycobacterial compounds by screening a pharmaceutical small-molecule library against non-replicating *Mycobacterium tuberculosis*. *ACS Infectious Diseases*. 2015
136. Deb C, Lee CM, Dubey VS, Daniel J, Abomoelak B, Sirakova TD, et al. A novel in vitro multiple-stress dormancy model for *Mycobacterium tuberculosis* generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS One*. 2009; 4(6):e6077. PubMed PMID: 19562030; PubMed Central PMCID: PMC2698117. [PubMed: 19562030]
137. Deris JB, Kim M, Zhang Z, Okano H, Hermesen R, Groisman A, et al. The innate growth bistability and fitness landscapes of antibiotic-resistant bacteria. *Science*. 2013; 342(6162): 1237435. PubMed PMID: 24288338; PubMed Central PMCID: PMC4059556. [PubMed: 24288338]
138. Salina EG, Waddell SJ, Hoffmann N, Rosenkrands I, Butcher PD, Kaprelyants AS. Potassium availability triggers *Mycobacterium tuberculosis* transition to, resuscitation from, non-culturable (dormant) states. *Open biology*. 2014; 4(10) PubMed PMID: 25320096; PubMed Central PMCID: PMC4221891.
139. Ignatov DV, Salina EG, Fursov MV, Skvortsov TA, Azhikina TL, Kaprelyants AS. Dormant non-culturable *Mycobacterium tuberculosis* retains stable low-abundant mRNA. *BMC genomics*. 2015; 16:954. PubMed PMID: 26573524; PubMed Central PMCID: PMC4647672. [PubMed: 26573524]
140. Kazius J, McGuire R, Bursi R. Derivation and validation of toxicophores for mutagenicity prediction. *J Med Chem*. 2005; 48(1):312–320. PubMed PMID: 15634026. [PubMed: 15634026]
141. Baell JB. Observations on screening-based research and some concerning trends in the literature. *Future medicinal chemistry*. 2010; 2(10):1529–1546. PubMed PMID: 21426147. [PubMed: 21426147]
142. Gold B, Deng H, Bryk R, Vargas D, Eliezer D, Roberts J, et al. Identification of a copper-binding metallothionein in pathogenic mycobacteria. *Nat Chem Biol*. 2008; 4(10):609–616. Epub 2008/08/30. PubMed PMID: 18724363; PubMed Central PMCID: PMC2749609. [PubMed: 18724363]
143. Kozikowski BA, Burt TM, Tirey DA, Williams LE, Kuzmak BR, Stanton DT, et al. The effect of freeze/thaw cycles on the stability of compounds in DMSO. *J Biomol Screen*. 2003; 8(2):210–215. PubMed PMID: 12844443. [PubMed: 12844443]
144. Baillargeon P, Scampavia L, Einsteder R, Hodder P. Monitoring of HTS compound library quality via a high-resolution image acquisition and processing instrument. *Journal of laboratory automation*. 2011; 16(3):197–203. PubMed PMID: 21609702; PubMed Central PMCID: PMC3417353. [PubMed: 21609702]
145. Di L, Kerns EH. Biological assay challenges from compound solubility: strategies for bioassay optimization. *Drug discovery today*. 2006; 11(9-10):446–451. PubMed PMID: 16635808. [PubMed: 16635808]
146. Ekins S, Kaneko T, Lipinski CA, Bradford J, Dole K, Spektor A, et al. Analysis and hit filtering of a very large library of compounds screened against *Mycobacterium tuberculosis*. *Molecular bioSystems*. 2010; 6(11):2316–2324. PubMed PMID: 20835433. [PubMed: 20835433]

147. Gold B, Smith R, Nguyen Q, Roberts J, Ling Y, Lopez Quezada L, et al. Novel cephalosporins selectively active on non-replicating *Mycobacterium tuberculosis*. J Med Chem. 2016 PubMed PMID: 27144688.
148. Williams K, Minkowski A, Amoabeng O, Peloquin CA, Taylor D, Andries K, et al. Sterilizing activities of novel combinations lacking first- and second-line drugs in a murine model of tuberculosis. Antimicrob Agents Chemother. 2012; 56(6):3114–3120. PubMed PMID: 22470112; PubMed Central PMCID: PMC3370712. [PubMed: 22470112]
149. Ibrahim M, Truffot-Pernot C, Andries K, Jarlier V, Veziris N. Sterilizing activity of R207910 (TMC207)-containing regimens in the murine model of tuberculosis. Am J Respir Crit Care Med. 2009; 180(6):553–557. PubMed PMID: 19590024. [PubMed: 19590024]
150. Diacon AH, Pym A, Grobusch M, Patientia R, Rustonjee R, Page-Shipp L, et al. The diarylquinoline TMC207 for multidrug-resistant tuberculosis. N Engl J Med. 2009; 360(23):2397–405. PubMed PMID: 19494215. [PubMed: 19494215]
151. Hohman M, Gregory K, Chibale K, Smith PJ, Ekins S, Bunin B. Novel web-based tools combining chemistry informatics, biology and social networks for drug discovery. Drug discovery today. 2009; 14(5-6):261–270. PubMed PMID: 19231313. [PubMed: 19231313]
152. Koul A, Vranckx L, Dendouga N, Balemans W, Van den Wyngaert I, Vergauwen K, et al. Diarylquinolines are bactericidal for dormant mycobacteria as a result of disturbed ATP homeostasis. J Biol Chem. 2008; 283(37):25273–25280. PubMed PMID: 18625705. [PubMed: 18625705]
153. Heifets, LB., Cynamon, MH. Drug susceptibility in the chemotherapy of mycobacterial infections. Boca Raton, Fla.: CRC Press; 1991. p. 212
154. Brotz-Oesterhelt H, Beyer D, Kroll HP, Endermann R, Ladel C, Schroeder W, et al. Dysregulation of bacterial proteolytic machinery by a new class of antibiotics. Nat Med. 2005; 11(10):1082–1087. PubMed PMID: 16200071. [PubMed: 16200071]
155. Conlon BP, Nakayasu ES, Fleck LE, LaFleur MD, Isabella VM, Coleman K, et al. Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature. 2013; 503(7476):365–370. PubMed PMID: 24226776; PubMed Central PMCID: PMC4031760. [PubMed: 24226776]
156. Tian J, Bryk R, Shi S, Erdjument-Bromage H, Tempst P, Nathan C. *Mycobacterium tuberculosis* appears to lack alpha-ketoglutarate dehydrogenase and encodes pyruvate dehydrogenase in widely separated genes. Mol Microbiol. 2005; 57(3):859–868. PubMed PMID: 16045627. [PubMed: 16045627]
157. Bryk R, Lima CD, Erdjument-Bromage H, Tempst P, Nathan C. Metabolic enzymes of mycobacteria linked to antioxidant defense by a thioredoxin-like protein. Science. 2002; 295(5557):1073–1077. PubMed PMID: 11799204. [PubMed: 11799204]
158. Ehrt S, Schnappinger D, Bekiranov S, Drenkow J, Shi S, Gingeras TR, et al. Reprogramming of the macrophage transcriptome in response to interferon-gamma and *Mycobacterium tuberculosis*: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. J Exp Med. 2001; 194(8):1123–1140. PubMed PMID: 11602641. [PubMed: 11602641]
159. Rao SP, Alonso S, Rand L, Dick T, Pethe K. The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A. 2008; 105(33):11945–11950. PubMed PMID: 18697942; PubMed Central PMCID: PMC2575262. [PubMed: 18697942]
160. Singh R, Manjunatha U, Boshoff HI, Ha YH, Niyomrattanakit P, Ledwidge R, et al. PA-824 kills nonreplicating *Mycobacterium tuberculosis* by intracellular NO release. Science. 2008; 322(5906):1392–1395. PubMed PMID: 19039139; PubMed Central PMCID: PMC2723733. [PubMed: 19039139]
161. Khan SR, Singh S, Roy KK, Akhtar MS, Saxena AK, Krishnan MY. Biological evaluation of novel substituted chloroquinolines targeting mycobacterial ATP synthase. International journal of antimicrobial agents. 2013; 41(1):41–46. PubMed PMID: 23141113. [PubMed: 23141113]
162. Herbert D, Paramasivan CN, Venkatesan P, Kubendiran G, Prabhakar R, Mitchison DA. Bactericidal action of ofloxacin, sulbactam-ampicillin, rifampin, and isoniazid on logarithmic- and stationary-phase cultures of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1996; 40(10):2296–2299. PubMed PMID: 8891133; PubMed Central PMCID: PMC163523. [PubMed: 8891133]

163. Hu Y, Coates AR, Mitchison DA. Sterilizing activities of fluoroquinolones against rifampin-tolerant populations of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2003; 47(2): 653–657. PubMed PMID: 12543673; PubMed Central PMCID: PMC151758. [PubMed: 12543673]
164. Hu Y, Coates A. Nonmultiplying bacteria are profoundly tolerant to antibiotics. *Handbook of experimental pharmacology*. 2012; (211):99–119. PubMed PMID: 23090598.
165. Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, Winkler H, et al. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science*. 2005; 307(5707):223–227. PubMed PMID: 15591164. [PubMed: 15591164]
166. Gengenbacher M, Rao SP, Pethe K, Dick T. Nutrient-starved, non-replicating *Mycobacterium tuberculosis* requires respiration, ATP synthase and isocitrate lyase for maintenance of ATP homeostasis and viability. *Microbiology*. 2010; 156(Pt 1):81–87. Epub 2009/10/03. PubMed PMID: 19797356. [PubMed: 19797356]
167. Diacon AH, Dawson R, von Groote-Bidlingmaier F, Symons G, Venter A, Donald PR, et al. 14-day bactericidal activity of PA-824, bedaquiline, pyrazinamide, and moxifloxacin combinations: a randomised trial. *Lancet*. 2012; 380(9846):986–993. PubMed PMID: 22828481. [PubMed: 22828481]
168. Zhang Y. The magic bullets and tuberculosis drug targets. *Annu Rev Pharmacol Toxicol*. 2005; 45:529–564. PubMed PMID: 15822188. [PubMed: 15822188]
169. Tan MP, Sequeira P, Lin WW, Phong WY, Cliff P, Ng SH, et al. Nitrate respiration protects hypoxic *Mycobacterium tuberculosis* against acid- and reactive nitrogen species stresses. *PLoS One*. 2010; 5(10):e13356. Epub 2010/11/05. PubMed PMID: 21048946; PubMed Central PMCID: PMC2965054. [PubMed: 21048946]
170. Eoh H, Rhee KY. Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. 2013; 110(16):6554–6559. PubMed PMID: 23576728; PubMed Central PMCID: PMC3631649. [PubMed: 23576728]
171. Huang Q, Chen ZF, Li YY, Zhang Y, Ren Y, Fu Z, et al. Nutrient-starved incubation conditions enhance pyrazinamide activity against *Mycobacterium tuberculosis*. *Chemotherapy*. 2007; 53(5): 338–343. PubMed PMID: 17785970. [PubMed: 17785970]
172. Zimhony O, Vilcheze C, Arai M, Welch JT, Jacobs WR Jr. Pyrazinoic acid and its n-propyl ester inhibit fatty acid synthase type I in replicating tubercle bacilli. *Antimicrob Agents Chemother*. 2007; 51(2):752–754. PubMed PMID: 17101678; PubMed Central PMCID: PMC1797748. [PubMed: 17101678]
173. Zimhony O, Cox JS, Welch JT, Vilcheze C, Jacobs WR Jr. Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FAS I) of *Mycobacterium tuberculosis*. *Nat Med*. 2000; 6(9):1043–1047. PubMed PMID: 10973326. [PubMed: 10973326]
174. Boshoff HI, Mizrahi V, Barry CE 3rd. Effects of pyrazinamide on fatty acid synthesis by whole mycobacterial cells and purified fatty acid synthase I. *J Bacteriol*. 2002; 184(8):2167–2172. PubMed PMID: 11914348; PubMed Central PMCID: PMC134955. [PubMed: 11914348]
175. Shi W, Zhang X, Jiang X, Yuan H, Lee JS, Barry CE 3rd, et al. Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*. *Science*. 2011; 333(6049):1630–1632. PubMed PMID: 21835980; PubMed Central PMCID: PMC3502614. [PubMed: 21835980]
176. Shi W, Chen J, Feng J, Cui P, Zhang S, Weng X, et al. Aspartate decarboxylase (PanD) as a new target of pyrazinamide in *Mycobacterium tuberculosis*. *Emerging microbes & infections*. 2014; 3(8):e58. PubMed PMID: 26038753; PubMed Central PMCID: PMC4150287.
177. Dillon NA, Peterson ND, Rosen BC, Baughn AD. Pantothenate and pantetheine antagonize the antitubercular activity of pyrazinamide. *Antimicrob Agents Chemother*. 2014; 58(12):7258–7263. PubMed PMID: 25246400; PubMed Central PMCID: PMC4249577. [PubMed: 25246400]
178. Sarathy J, Dartois V, Dick T, Gengenbacher M. Reduced drug uptake in phenotypically resistant nutrient-starved nonreplicating *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2013; 57(4):1648–1653. PubMed PMID: 23335744; PubMed Central PMCID: PMC3623341. [PubMed: 23335744]

179. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature reviews Drug discovery*. 2007; 6(1):29–40. PubMed PMID: 17159923. [PubMed: 17159923]
180. Rhee KY, Erdjument-Bromage H, Tempst P, Nathan CF. S-nitroso proteome of *Mycobacterium tuberculosis*: Enzymes of intermediary metabolism and antioxidant defense. *Proc Natl Acad Sci U S A*. 2005; 102(2):467–472. PubMed PMID: 15626759. [PubMed: 15626759]
181. Vilcheze C, Baughn AD, Tufariello J, Leung LW, Kuo M, Basler CF, et al. Novel inhibitors of InhA efficiently kill *Mycobacterium tuberculosis* under aerobic and anaerobic conditions. *Antimicrob Agents Chemother*. 2011; 55(8):3889–3898. Epub 2011/06/02. PubMed PMID: 21628538; PubMed Central PMCID: PMC3147652. [PubMed: 21628538]
182. Martinez-Hoyos M, Perez-Herran E, Gulten G, Encinas L, Alvarez-Gomez D, Alvarez E, et al. Antitubercular drugs for an old target: GSK693 as a promising InhA direct inhibitor. *EBioMedicine*. 2016; 8:291–301. PubMed PMID: 27428438; PubMed Central PMCID: PMC4919555. [PubMed: 27428438]
183. Stover CK, Warrenner P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH, et al. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature*. 2000; 405(6789):962–966. Epub 2000/07/06. PubMed PMID: 10879539. [PubMed: 10879539]
184. Manjunatha U, Boshoff HI, Barry CE. The mechanism of action of PA-824: Novel insights from transcriptional profiling. *Communicative & integrative biology*. 2009; 2(3):215–218. PubMed PMID: 19641733; PubMed Central PMCID: PMC2717523. [PubMed: 19641733]
185. Machaba KE, Cele FN, Mhlongo NN, Soliman ME. Sliding Clamp of DNA Polymerase III as a Drug Target for TB Therapy: Comprehensive Conformational and Binding Analysis from Molecular Dynamic Simulations. *Cell biochemistry and biophysics*. 2016 PubMed PMID: 27651172.
186. Kling A, Lukat P, Almeida DV, Bauer A, Fontaine E, Sordello S, et al. Antibiotics. Targeting DnaN for tuberculosis therapy using novel griselimycins. *Science*. 2015; 348(6239):1106–1112. PubMed PMID: 26045430. [PubMed: 26045430]
187. Herrmann J, Lukezic T, Kling A, Baumann S, Huttel S, Petkovic H, et al. Strategies for the Discovery and Development of New Antibiotics from Natural Products: Three Case Studies. *Current topics in microbiology and immunology*. 2016 PubMed PMID: 27738913.
188. Higgins PG, Fluit AC, Schmitz FJ. Fluoroquinolones: structure and target sites. *Current drug targets*. 2003; 4(2):181–190. PubMed PMID: 12558069. [PubMed: 12558069]
189. Hu YM, Butcher PD, Sole K, Mitchison DA, Coates AR. Protein synthesis is shutdown in dormant *Mycobacterium tuberculosis* and is reversed by oxygen or heat shock. *FEMS Microbiol Lett*. 1998; 158(1):139–145. PubMed PMID: 9453166. [PubMed: 9453166]
190. Lee RE, Hurdle JG, Liu J, Bruhn DF, Matt T, Scherman MS, et al. Spectinamides: a new class of semisynthetic antituberculosis agents that overcome native drug efflux. *Nat Med*. 2014; 20(2): 152–158. PubMed PMID: 24464186; PubMed Central PMCID: PMC3972818. [PubMed: 24464186]
191. Jarlier V, Nikaido H. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonae*. *J Bacteriol*. 1990; 172(3):1418–1423. PubMed PMID: 2307653; PubMed Central PMCID: PMC208614. [PubMed: 2307653]
192. Kasik JE. The Nature of Mycobacterial Penicillinase. *Am Rev Respir Dis*. 1965; 91:117–119. PubMed PMID: 14259993. [PubMed: 14259993]
193. Jarlier V, Gutmann L, Nikaido H. Interplay of cell wall barrier and beta-lactamase activity determines high resistance to beta-lactam antibiotics in *Mycobacterium chelonae*. *Antimicrob Agents Chemother*. 1991; 35(9):1937–1939. PubMed PMID: 1952873; PubMed Central PMCID: PMC245299. [PubMed: 1952873]
194. Finch R. Beta-lactam antibiotics and mycobacteria. *J Antimicrob Chemother*. 1986; 18(1):6–8. PubMed PMID: 3093446. [PubMed: 3093446]
195. Hugonnet JE, Tremblay LW, Boshoff HI, Barry CE 3rd, Blanchard JS. Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. *Science*. 2009; 323(5918):1215–1218. Epub 2009/03/03. PubMed PMID: 19251630; PubMed Central PMCID: PMC2679150. [PubMed: 19251630]

196. Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A. The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. *J Gen Microbiol*. 1986; 132(5):1297–1304. PubMed PMID: 3534137. [PubMed: 3534137]
197. Schoonmaker MK, Bishai WR, Lamichhane G. Nonclassical transpeptidases of *Mycobacterium tuberculosis* alter cell size, morphology, the cytosolic matrix, protein localization, virulence, and resistance to beta-lactams. *J Bacteriol*. 2014; 196(7):1394–1402. PubMed PMID: 24464457; PubMed Central PMCID: PMC3993333. [PubMed: 24464457]
198. Vollmer W, Holtje JV. The architecture of the murein (peptidoglycan) in gram-negative bacteria: vertical scaffold or horizontal layer(s)? *J Bacteriol*. 2004; 186(18):5978–5987. PubMed PMID: 15342566; PubMed Central PMCID: PMC515156. [PubMed: 15342566]
199. Gupta R, Lavollay M, Mainardi JL, Arthur M, Bishai WR, Lamichhane G. The *Mycobacterium tuberculosis* protein LdtMt2 is a nonclassical transpeptidase required for virulence and resistance to amoxicillin. *Nat Med*. 2010; 16(4):466–469. PubMed PMID: 20305661; PubMed Central PMCID: PMC2851841. [PubMed: 20305661]
200. Diacon AH, van der Merwe L, Barnard M, von Groote-Bidlingmaier F, Lange C, García-Basteiro AL, et al. β -Lactams against Tuberculosis — New Trick for an Old Dog? *New England Journal of Medicine*. 0(0) null.
201. Solapure S, Dinesh N, Shandil R, Ramachandran V, Sharma S, Bhattacharjee D, et al. In vitro and in vivo efficacy of beta-lactams against replicating and slowly growing/nonreplicating *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2013; 57(6):2506–2510. PubMed PMID: 23507276; PubMed Central PMCID: PMC3716166. [PubMed: 23507276]
202. Borrelli S, Zandberg WF, Mohan S, Ko M, Martinez-Gutierrez F, Partha SK, et al. Antimycobacterial activity of UDP-galactopyranose mutase inhibitors. *International journal of antimicrobial agents*. 2010; 36(4):364–368. PubMed PMID: 20678902. [PubMed: 20678902]
203. Engohang-Ndong J. Antimycobacterial drugs currently in Phase II clinical trials and preclinical phase for tuberculosis treatment. *Expert opinion on investigational drugs*. 2012; 21(12):1789–1800. PubMed PMID: 22991970. [PubMed: 22991970]
204. Siricilla S, Mitachi K, Wan B, Franzblau SG, Kurosu M. Discovery of a capuramycin analog that kills nonreplicating *Mycobacterium tuberculosis* and its synergistic effects with translocase I inhibitors. *The Journal of antibiotics*. 2015; 68(4):271–278. PubMed PMID: 25269459; PubMed Central PMCID: PMC4382465. [PubMed: 25269459]
205. Ishizaki Y, Hayashi C, Inoue K, Igarashi M, Takahashi Y, Pujari V, et al. Inhibition of the first step in synthesis of the mycobacterial cell wall core, catalyzed by the GlcNAc-1-phosphate transferase WecA, by the novel caprazamycin derivative CPZEN-45. *J Biol Chem*. 2013; 288(42):30309–30319. PubMed PMID: 23986448; PubMed Central PMCID: PMC3798496. [PubMed: 23986448]
206. Singh S, Roy KK, Khan SR, Kashyap VK, Sharma A, Jaiswal S, et al. Novel, potent, orally bioavailable and selective mycobacterial ATP synthase inhibitors that demonstrated activity against both replicating and non-replicating *M. tuberculosis*. *Bioorg Med Chem*. 2015; 23(4):742–752. PubMed PMID: 25614114. [PubMed: 25614114]
207. Danelishvili L, Wu M, Young LS, Bermudez LE. Genomic approach to identifying the putative target of and mechanisms of resistance to mefloquine in mycobacteria. *Antimicrob Agents Chemother*. 2005; 49(9):3707–3714. PubMed PMID: 16127044; PubMed Central PMCID: PMC1195445. [PubMed: 16127044]
208. Martin-Galiano AJ, Gorgojo B, Kunin CM, de la Campa AG. Mefloquine and new related compounds target the F(0) complex of the F(0)F(1) H(+)-ATPase of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother*. 2002; 46(6):1680–1687. PubMed PMID: 12019076; PubMed Central PMCID: PMC127268. [PubMed: 12019076]
209. Hongmanee P, Rukseree K, Buabut B, Somsri B, Palittapongarnpim P. In vitro activities of cloxyquin (5-chloroquinolin-8-ol) against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2007; 51(3):1105–1106. PubMed PMID: 17178795; PubMed Central PMCID: PMC1803129. [PubMed: 17178795]
210. Tison F. [The remarkable effect of a combination of iodochloroxyquinoline with a subactive dose of streptomycin on experimental tuberculosis in guinea pigs]. *Annales de l'Institut Pasteur*. 1952; 83(2):275–276. Epub 1952/08/01. PubMed PMID: 12986381.

211. Shah S, Dalecki AG, Malalasekera AP, Crawford CL, Michalek SM, Kutsch O, et al. 8-Hydroxyquinolines are boosting-agents of copper related toxicity in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2016 PubMed PMID: 27431227.
212. Mao J, Wang Y, Wan B, Kozikowski AP, Franzblau SG. Design, synthesis, and pharmacological evaluation of mefloquine-based ligands as novel antituberculosis agents. ChemMedChem. 2007; 2(11):1624–1630. PubMed PMID: 17680579. [PubMed: 17680579]
213. Mao J, Yuan H, Wang Y, Wan B, Pieroni M, Huang Q, et al. From serendipity to rational antituberculosis drug discovery of mefloquine-isoxazole carboxylic acid esters. J Med Chem. 2009; 52(22):6966–6978. PubMed PMID: 19863050. [PubMed: 19863050]
214. Jayaprakash S, Iso Y, Wan B, Franzblau SG, Kozikowski AP. Design, synthesis, and SAR studies of mefloquine-based ligands as potential antituberculosis agents. ChemMedChem. 2006; 1(6):593–597. PubMed PMID: 16892398. [PubMed: 16892398]
215. Lilienkampf A, Mao J, Wan B, Wang Y, Franzblau SG, Kozikowski AP. Structure-activity relationships for a series of quinoline-based compounds active against replicating and nonreplicating *Mycobacterium tuberculosis*. J Med Chem. 2009; 52(7):2109–2118. PubMed PMID: 19271749. [PubMed: 19271749]
216. Lilienkampf A, Pieroni M, Wan B, Wang Y, Franzblau SG, Kozikowski AP. Rational design of 5-phenyl-3-isoxazolecarboxylic acid ethyl esters as growth inhibitors of *Mycobacterium tuberculosis*. a potent and selective series for further drug development. J Med Chem. 2010; 53(2):678–688. PubMed PMID: 20000577. [PubMed: 20000577]
217. Saxena AK, Roy KK, Singh S, Vishnoi SP, Kumar A, Kashyap VK, et al. Identification and characterisation of small-molecule inhibitors of Rv3097c-encoded lipase (LipY) of *Mycobacterium tuberculosis* that selectively inhibit growth of bacilli in hypoxia. International journal of antimicrobial agents. 2013; 42(1):27–35. PubMed PMID: 23684389. [PubMed: 23684389]
218. Low KL, Rao PS, Shui G, Bendt AK, Pethe K, Dick T, et al. Triacylglycerol utilization is required for regrowth of in vitro hypoxic nonreplicating *Mycobacterium bovis* bacillus Calmette-Guerin. J Bacteriol. 2009; 191(16):5037–5043. PubMed PMID: 19525349; PubMed Central PMCID: PMC2725574. [PubMed: 19525349]
219. Darwin KH, Ehrt S, Gutierrez-Ramos JC, Weich N, Nathan CF. The proteasome of *Mycobacterium tuberculosis* is required for resistance to nitric oxide. Science. 2003; 302(5652):1963–1966. PubMed PMID: 14671303. [PubMed: 14671303]
220. Fay A, Glickman MS. An essential nonredundant role for mycobacterial DnaK in native protein folding. PLoS genetics. 2014; 10(7):e1004516. PubMed PMID: 25058675; PubMed Central PMCID: PMC4109909. [PubMed: 25058675]
221. Raju RM, Unnikrishnan M, Rubin DH, Krishnamoorthy V, Kandror O, Akopian TN, et al. *Mycobacterium tuberculosis* ClpP1 and ClpP2 function together in protein degradation and are required for viability in vitro and during infection. PLoS pathogens. 2012; 8(2):e1002511. PubMed PMID: 22359499; PubMed Central PMCID: PMC3280978. [PubMed: 22359499]
222. Schmitt EK, Riwanto M, Sambandamurthy V, Roggo S, Miault C, Zwingelstein C, et al. The natural product cyclomarin kills *Mycobacterium tuberculosis* by targeting the ClpC1 subunit of the caseinolytic protease. Angewandte Chemie. 2011; 50(26):5889–5891. PubMed PMID: 21563281. [PubMed: 21563281]
223. Lee H, Suh JW. Anti-tuberculosis lead molecules from natural products targeting *Mycobacterium tuberculosis* ClpC1. Journal of industrial microbiology & biotechnology. 2016; 43(2-3):205–212. PubMed PMID: 26586403. [PubMed: 26586403]
224. Lear S, Munshi T, Hudson AS, Hatton C, Clardy J, Mosely JA, et al. Total chemical synthesis of lassomycin and lassomycin-amide. Organic & biomolecular chemistry. 2016; 14(19):4534–4541. PubMed PMID: 27101411. [PubMed: 27101411]
225. Gavrish E, Sit CS, Cao S, Kandror O, Spoering A, Peoples A, et al. Lassomycin, a ribosomally synthesized cyclic peptide, kills mycobacterium tuberculosis by targeting the ATP-dependent protease ClpC1P2. Chem Biol. 2014; 21(4):509–518. PubMed PMID: 24684906; PubMed Central PMCID: PMC4060151. [PubMed: 24684906]
226. Gao W, Kim JY, Anderson JR, Akopian T, Hong S, Jin YY, et al. The cyclic peptide ecumicin targeting ClpC1 is active against *Mycobacterium tuberculosis* in vivo. Antimicrob Agents

- Chemother. 2015; 59(2):880–889. PubMed PMID: 25421483; PubMed Central PMCID: PMC4335914. [PubMed: 25421483]
227. Gandotra S, Schnappinger D, Monteleone M, Hillen W, Ehrt S. In vivo gene silencing identifies the *Mycobacterium tuberculosis* proteasome as essential for the bacteria to persist in mice. *Nat Med*. 2007; 13(12):1515–1520. Epub 2007/12/07. PubMed PMID: 18059281. [PubMed: 18059281]
 228. Gandotra S, Lebron MB, Ehrt S. The *Mycobacterium tuberculosis* proteasome active site threonine is essential for persistence yet dispensable for replication and resistance to nitric oxide. *PLoS pathogens*. 2010; 6(8):e1001040. PubMed PMID: 20711362; PubMed Central PMCID: PMC2920845. [PubMed: 20711362]
 229. Lin G, Chidawanyika T, Tsu C, Warriar T, Vaubourgeix J, Blackburn C, et al. N,C-Capped dipeptides with selectivity for mycobacterial proteasome over human proteasomes: role of S3 and S1 binding pockets. *Journal of the American Chemical Society*. 2013; 135(27):9968–9971. PubMed PMID: 23782398; PubMed Central PMCID: PMC3773049. [PubMed: 23782398]
 230. Russo F, Gising J, Akerbladh L, Roos AK, Naworyta A, Mowbray SL, et al. Optimization and Evaluation of 5-Styryl-Oxathiazol-2-one *Mycobacterium tuberculosis* Proteasome Inhibitors as Potential Antitubercular Agents. *ChemistryOpen*. 2015; 4(3):342–362. PubMed PMID: 26246997; PubMed Central PMCID: PMC4522185. [PubMed: 26246997]
 231. Timm J, Post FA, Bekker LG, Walther GB, Wainwright HC, Manganelli R, et al. Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. *Proc Natl Acad Sci U S A*. 2003; 100(24):14321–14326. PubMed PMID: 14623960; PubMed Central PMCID: PMC283590. [PubMed: 14623960]
 232. Wolschendorf F, Ackart D, Shrestha TB, Hascall-Dove L, Nolan S, Lamichhane G, et al. Copper resistance is essential for virulence of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. 2011; 108(4):1621–1626. PubMed PMID: 21205886; PubMed Central PMCID: PMC3029754. [PubMed: 21205886]
 233. MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science*. 2003; 302(5645):654–659. PubMed PMID: 14576437. [PubMed: 14576437]
 234. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci U S A*. 1997; 94(10):5243–5248. PubMed PMID: 9144222. [PubMed: 9144222]
 235. Marrero J, Rhee KY, Schnappinger D, Pethe K, Ehrt S. Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection. *Proc Natl Acad Sci U S A*. 2010; 107(21):9819–9824. PubMed PMID: 20439709; PubMed Central PMCID: PMC2906907. [PubMed: 20439709]
 236. Gomez JE, McKinney JD. M. tuberculosis persistence, latency, and drug tolerance. *Tuberculosis (Edinb)*. 2004; 84(1-2):29–44. Epub 2003/12/13. PubMed PMID: 14670344. [PubMed: 14670344]
 237. Manina G, Dhar N, McKinney JD. Stress and host immunity amplify *Mycobacterium tuberculosis* phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe*. 2015; 17(1):32–46. PubMed PMID: 25543231. [PubMed: 25543231]
 238. Munoz-Elias EJ, McKinney JD. *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat Med*. 2005; 11(6):638–644. Epub 2005/05/17. PubMed PMID: 15895072; PubMed Central PMCID: PMC1464426. [PubMed: 15895072]
 239. Munoz-Elias EJ, McKinney JD. Carbon metabolism of intracellular bacteria. *Cell Microbiol*. 2006; 8(1):10–22. Epub 2005/12/22. PubMed PMID: 16367862. [PubMed: 16367862]
 240. Berney M, Berney-Meyer L, Wong KW, Chen B, Chen M, Kim J, et al. Essential roles of methionine and S-adenosylmethionine in the autarkic lifestyle of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. 2015; 112(32):10008–10013. PubMed PMID: 26221021; PubMed Central PMCID: PMC4538671. [PubMed: 26221021]
 241. Hondalus MK, Bardarov S, Russell R, Chan J, Jacobs WR Jr, Bloom BR. Attenuation of and protection induced by a leucine auxotroph of *Mycobacterium tuberculosis*. *Infect Immun*. 2000;

- 68(5):2888–2898. Epub 2000/04/18. PubMed PMID: 10768986; PubMed Central PMCID: PMC97501. [PubMed: 10768986]
242. Sambandamurthy VK, Wang X, Chen B, Russell RG, Derrick S, Collins FM, et al. A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. *Nat Med.* 2002; 8(10):1171–1174. Epub 2002/09/10. PubMed PMID: 12219086. [PubMed: 12219086]
 243. Rodriguez GM, Smith I. Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. *Mol Microbiol.* 2003; 47(6):1485–1494. PubMed PMID: 12622807. [PubMed: 12622807]
 244. Gould TA, van de Langemheen H, Munoz-Elias EJ, McKinney JD, Sacchettini JC. Dual role of isocitrate lyase 1 in the glyoxylate and methylcitrate cycles in *Mycobacterium tuberculosis*. *Mol Microbiol.* 2006; 61(4):940–947. Epub 2006/08/02. PubMed PMID: 16879647. [PubMed: 16879647]
 245. Walters SB, Dubnau E, Kolesnikova I, Laval F, Daffe M, Smith I. The *Mycobacterium tuberculosis* PhoPR two-component system regulates genes essential for virulence and complex lipid biosynthesis. *Mol Microbiol.* 2006; 60(2):312–330. Epub 2006/04/01. PubMed PMID: 16573683. [PubMed: 16573683]
 246. Miner MD, Chang JC, Pandey AK, Sassetti CM, Sherman DR. Role of cholesterol in *Mycobacterium tuberculosis* infection. *Indian J Exp Biol.* 2009; 47(6):407–411. Epub 2009/07/29. PubMed PMID: 19634704. [PubMed: 19634704]
 247. Belton M, Brilha S, Manavaki R, Mauri F, Nijran K, Hong YT, et al. Hypoxia and tissue destruction in pulmonary TB. *Thorax.* 2016 PubMed PMID: 27245780.
 248. Sperka T, Pitlik J, Bagossi P, Tozser J. Beta-lactam compounds as apparently uncompetitive inhibitors of HIV-1 protease. *Bioorganic & medicinal chemistry letters.* 2005; 15(12):3086–3090. PubMed PMID: 15893929. [PubMed: 15893929]
 249. Powers JC, Asgian JL, Ekici OD, James KE. Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chemical reviews.* 2002; 102(12):4639–4750. PubMed PMID: 12475205. [PubMed: 12475205]
 250. Paetzel M, Dalbey RE, Strynadka NC. Crystal structure of a bacterial signal peptidase in complex with a beta-lactam inhibitor. *Nature.* 1998; 396(6707):186–190. PubMed PMID: 9823901. [PubMed: 9823901]
 251. Baranowski C, Rubin EJ. Could Killing Bacterial Subpopulations Hit Tuberculosis out of the Park? *J Med Chem.* 2016; 59(13):6025–606. PubMed PMID: 27322073. [PubMed: 27322073]
 252. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science.* 1999; 284(5418):1318–1322. PubMed PMID: 10334980. [PubMed: 10334980]
 253. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol.* 2004; 2(2):95–108. PubMed PMID: 15040259. [PubMed: 15040259]
 254. Ojha AK, Baughn AD, Sambandan D, Hsu T, Trivelli X, Guerardel Y, et al. Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol Microbiol.* 2008; 69(1):164–174. PubMed PMID: 18466296; PubMed Central PMCID: PMC2615189. [PubMed: 18466296]
 255. Purkayastha A, McCue LA, McDonough KA. Identification of a *Mycobacterium tuberculosis* putative classical nitroreductase gene whose expression is coregulated with that of the *acrA* gene within macrophages, in standing versus shaking cultures, and under low oxygen conditions. *Infect Immun.* 2002; 70(3):1518–1529. PubMed PMID: 11854240; PubMed Central PMCID: PMC127740. [PubMed: 11854240]
 256. Williams EM, Little RF, Mowday AM, Rich MH, Chan-Hyams JV, Copp JN, et al. Nitroreductase gene-directed enzyme prodrug therapy: insights and advances toward clinical utility. *Biochem J.* 2015; 471(2):131–153. PubMed PMID: 26431849. [PubMed: 26431849]
 257. Viode C, Bettache N, Cenas N, Krauth-Siegel RL, Chauviere G, Bakalara N, et al. Enzymatic reduction studies of nitroheterocycles. *Biochemical pharmacology.* 1999; 57(5):549–557. PubMed PMID: 9952319. [PubMed: 9952319]

258. McOsker CC, Fitzpatrick PM. Nitrofurantoin: mechanism of action and implications for resistance development in common uropathogens. *J Antimicrob Chemother.* 1994; 33(Suppl A): 23–30. PubMed PMID: 7928834. [PubMed: 7928834]
259. Murugasu-Oei B, Dick T. Bactericidal activity of nitrofurans against growing and dormant *Mycobacterium bovis* BCG. *J Antimicrob Chemother.* 2000; 46(6):917–919. PubMed PMID: 11102410. [PubMed: 11102410]
260. de Carvalho LP, Darby CM, Rhee KY, Nathan C. Nitazoxanide Disrupts Membrane Potential and Intrabacterial pH Homeostasis of *Mycobacterium tuberculosis*. *ACS medicinal chemistry letters.* 2011; 2(11):849–854. PubMed PMID: 22096616; PubMed Central PMCID: PMC3215101. [PubMed: 22096616]
261. Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, Sasaki H, et al. OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. *PLoS Med.* 2006; 3(11):e466. PubMed PMID: 17132069; PubMed Central PMCID: PMC1664607. [PubMed: 17132069]
262. Upton AM, Cho S, Yang TJ, Kim Y, Wang Y, Lu Y, et al. In vitro and in vivo activities of the nitroimidazole TBA-354 against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother.* 2015; 59(1):136–144. PubMed PMID: 25331696; PubMed Central PMCID: PMC4291341. [PubMed: 25331696]
263. Upton AM, McKinney JD. Role of the methylcitrate cycle in propionate metabolism and detoxification in *Mycobacterium smegmatis*. *Microbiology.* 2007; 153(Pt 12):3973–3982. Epub 2007/12/01. PubMed PMID: 18048912. [PubMed: 18048912]
264. Rakesh, Bruhn DF, Scherman MS, Woolhiser LK, Madhura DB, Maddox MM, et al. Pentacyclic nitrofurans with in vivo efficacy and activity against nonreplicating *Mycobacterium tuberculosis*. *PLoS One.* 2014; 9(2):e87909. PubMed PMID: 24505329; PubMed Central PMCID: PMC3914891. [PubMed: 24505329]
265. Debnath AK, Lopez de Compadre RL, Debnath G, Shusterman AJ, Hansch C. Structure-activity relationship of mutagenic aromatic and heteroaromatic nitro compounds. Correlation with molecular orbital energies and hydrophobicity. *J Med Chem.* 1991; 34(2):786–797. PubMed PMID: 1995902. [PubMed: 1995902]
266. Feng X, Zhu W, Schurig-Briccio LA, Lindert S, Shoen C, Hitchings R, et al. Anti-infectives targeting enzymes and the proton motive force. *Proc Natl Acad Sci U S A.* 2015; 112(51):E7073–E7082. PubMed PMID: 26644565; PubMed Central PMCID: PMC4697371. [PubMed: 26644565]
267. Lee IY, Gruber TD, Samuels A, Yun M, Nam B, Kang M, et al. Structure-activity relationships of antitubercular salicylanilides consistent with disruption of the proton gradient via proton shuttling. *Bioorg Med Chem.* 2013; 21(1):114–126. PubMed PMID: 23211970; PubMed Central PMCID: PMC3525752. [PubMed: 23211970]
268. Terada H. Uncouplers of oxidative phosphorylation. *Environmental health perspectives.* 1990; 87:213–218. PubMed PMID: 2176586; PubMed Central PMCID: PMC1567840. [PubMed: 2176586]
269. Williamson RL, Metcalf RL. Salicylanilides: a new group of active uncouplers of oxidative phosphorylation. *Science.* 1967; 158(3809):1694–1695. PubMed PMID: 4228706. [PubMed: 4228706]
270. Moreira W, Aziz DB, Dick T. Boromycin Kills Mycobacterial Persisters without Detectable Resistance. *Frontiers in microbiology.* 2016; 7:199. PubMed PMID: 26941723; PubMed Central PMCID: PMC4761863. [PubMed: 26941723]
271. Mukherjee D, Zou H, Liu S, Beuerman R, Dick T. Membrane-targeting AM-0016 kills mycobacterial persisters and shows low propensity for resistance development. *Future Microbiol.* 2016; 11:643–650. PubMed PMID: 27158932. [PubMed: 27158932]
272. Tyagi S, Ammerman NC, Li SY, Adamson J, Converse PJ, Swanson RV, et al. Clofazimine shortens the duration of the first-line treatment regimen for experimental chemotherapy of tuberculosis. *Proc Natl Acad Sci U S A.* 2015; 112(3):869–874. PubMed PMID: 25561537; PubMed Central PMCID: PMC4311815. [PubMed: 25561537]
273. Yano T, Kassovska-Bratinova S, Teh JS, Winkler J, Sullivan K, Isaacs A, et al. Reduction of clofazimine by mycobacterial type 2 NADH:quinone oxidoreductase: a pathway for the

generation of bactericidal levels of reactive oxygen species. *J Biol Chem.* 2011; 286(12):10276–10287. PubMed PMID: 21193400; PubMed Central PMCID: PMC3060482. [PubMed: 21193400]

274. Lechartier B, Cole ST. Mode of Action of Clofazimine and Combination Therapy with Benzothiazinones against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother.* 2015; 59(8):4457–4463. PubMed PMID: 25987624; PubMed Central PMCID: PMC4505229. [PubMed: 25987624]
275. Advani MJ, Siddiqui I, Sharma P, Reddy H. Activity of trifluoperazine against replicating, non-replicating and drug resistant *M. tuberculosis*. *PLoS One.* 2012; 7(8):e44245. PubMed PMID: 22952939; PubMed Central PMCID: PMC3432105. [PubMed: 22952939]
276. Jones PB, Parrish NM, Houston TA, Stapon A, Bansal NP, Dick JD, et al. A new class of antituberculosis agents. *J Med Chem.* 2000; 43(17):3304–3314. PubMed PMID: 10966749. [PubMed: 10966749]
277. Parrish NM, Houston T, Jones PB, Townsend C, Dick JD. In vitro activity of a novel antimycobacterial compound, N-octanesulfonylacetamide, and its effects on lipid and mycolic acid synthesis. *Antimicrob Agents Chemother.* 2001; 45(4):1143–1150. PubMed PMID: 11257028; PubMed Central PMCID: PMC90437. [PubMed: 11257028]
278. Parrish NM, Ko CG, Dick JD. Activity of DSA against anaerobically adapted *Mycobacterium bovis* BCG in vitro. *Tuberculosis (Edinb).* 2009; 89(4):325–327. PubMed PMID: 19520609; PubMed Central PMCID: PMC2760961. [PubMed: 19520609]
279. Parrish NM, Ko CG, Hughes MA, Townsend CA, Dick JD. Effect of n-octanesulphonylacetamide (OSA) on ATP and protein expression in *Mycobacterium bovis* BCG. *J Antimicrob Chemother.* 2004; 54(4):722–729. PubMed PMID: 15355939. [PubMed: 15355939]
280. Pethe K, Bifani P, Jang J, Kang S, Park S, Ahn S, et al. Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. *Nat Med.* 2013; 19(9):1157–1160. PubMed PMID: 23913123. [PubMed: 23913123]
281. Wallace KB, Starkov AA. Mitochondrial targets of drug toxicity. *Annu Rev Pharmacol Toxicol.* 2000; 40:353–388. PubMed PMID: 10836141. [PubMed: 10836141]
282. Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, et al. Persister formation in *Staphylococcus aureus* is associated with ATP depletion. *Nature microbiology.* 2016;1. PubMed PMID: 27398229; PubMed Central PMCID: PMC4932909.
283. Harbut MB, Vilcheze C, Luo X, Hensler ME, Guo H, Yang B, et al. Auranofin exerts broad-spectrum bactericidal activities by targeting thiol-redox homeostasis. *Proc Natl Acad Sci U S A.* 2015; 112(14):4453–4458. PubMed PMID: 25831516; PubMed Central PMCID: PMC4394260. [PubMed: 25831516]
284. Lin K, O'Brien KM, Trujillo C, Wang R, Wallach JB, Schnappinger D, et al. *Mycobacterium tuberculosis* Thioredoxin Reductase Is Essential for Thiol Redox Homeostasis but Plays a Minor Role in Antioxidant Defense. *PLoS pathogens.* 2016; 12(6):e1005675. PubMed PMID: 27249779; PubMed Central PMCID: PMC4889078. [PubMed: 27249779]
285. Malhotra V, Sharma D, Ramanathan VD, Shakila H, Saini DK, Chakravorty S, et al. Disruption of response regulator gene, devR, leads to attenuation in virulence of *Mycobacterium tuberculosis*. *FEMS Microbiol Lett.* 2004; 231(2):237–245. PubMed PMID: 14987770. [PubMed: 14987770]
286. Gupta RK, Thakur TS, Desiraju GR, Tyagi JS. Structure-based design of DevR inhibitor active against nonreplicating *Mycobacterium tuberculosis*. *J Med Chem.* 2009; 52(20):6324–6334. PubMed PMID: 19827833. [PubMed: 19827833]
287. Salina E, Ryabova O, Kaprelyants A, Makarov V. New 2-thiopyridines as potential candidates for killing both actively growing and dormant *Mycobacterium tuberculosis* cells. *Antimicrob Agents Chemother.* 2014; 58(1):55–60. PubMed PMID: 24126578; PubMed Central PMCID: PMC3910762. [PubMed: 24126578]
288. Moraski GC, Chang M, Villegas-Estrada A, Franzblau SG, Mollmann U, Miller MJ. Structure-activity relationship of new anti-tuberculosis agents derived from oxazoline and oxazole benzyl esters. *European journal of medicinal chemistry.* 2010; 45(5):1703–1716. PubMed PMID: 20116900; PubMed Central PMCID: PMC2843756. [PubMed: 20116900]

289. Villar R, Vicente E, Solano B, Perez-Silanes S, Aldana I, Maddry JA, et al. In vitro and in vivo antimycobacterial activities of ketone and amide derivatives of quinoxaline 1,4-di-N-oxide. *J Antimicrob Chemother.* 2008; 62(3):547–554. PubMed PMID: 18502817; PubMed Central PMCID: PMC2574959. [PubMed: 18502817]
290. Friedmann HC. From "butyribacterium" to "E. coli": an essay on unity in biochemistry. *Perspectives in biology and medicine.* 2004; 47(1):47–66. PubMed PMID: 15061168. [PubMed: 15061168]
291. Schnappinger D. Genetic Approaches to Facilitate Antibacterial Drug Development. *Cold Spring Harbor perspectives in medicine.* 2015; 5(7):a021139. PubMed PMID: 25680982. [PubMed: 25680982]
292. Kim JH, O'Brien KM, Sharma R, Boshoff HI, Rehren G, Chakraborty S, et al. A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence. *Proc Natl Acad Sci U S A.* 2013; 110(47):19095–19100. PubMed PMID: 24191058; PubMed Central PMCID: PMC3839782. [PubMed: 24191058]
293. Russell DG, Barry CE 3rd, Flynn JL. Tuberculosis: what we don't know can, and does, hurt us. *Science.* 2010; 328(5980):852–856. PubMed PMID: 20466922; PubMed Central PMCID: PMC2872107. [PubMed: 20466922]
294. Heifets L, Higgins M, Simon B. Pyrazinamide is not active against *Mycobacterium tuberculosis* residing in cultured human monocyte-derived macrophages. *Int J Tuberc Lung Dis.* 2000; 4(6): 491–495. PubMed PMID: 10864178. [PubMed: 10864178]
295. Rastogi N, Potar MC, David HL. Pyrazinamide is not effective against intracellularly growing *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother.* 1988; 32(2):287. PubMed PMID: 3129990; PubMed Central PMCID: PMC172157. [PubMed: 3129990]
296. Tsai MC, Chakravarty S, Zhu G, Xu J, Tanaka K, Koch C, et al. Characterization of the tuberculous granuloma in murine and human lungs: cellular composition and relative tissue oxygen tension. *Cell Microbiol.* 2006; 8(2):218–232. PubMed PMID: 16441433. [PubMed: 16441433]
297. Dhillon J, Allen BW, Hu YM, Coates AR, Mitchison DA. Metronidazole has no antibacterial effect in Cornell model murine tuberculosis. *Int J Tuberc Lung Dis.* 1998; 2(9):736–742. PubMed PMID: 9755928. [PubMed: 9755928]
298. Lanoix JP, Lenaerts AJ, Nuermberger EL. Heterogeneous disease progression and treatment response in a C3HeB/FeJ mouse model of tuberculosis. *Disease models & mechanisms.* 2015; 8(6):603–610. PubMed PMID: 26035868; PubMed Central PMCID: PMC4457036. [PubMed: 26035868]
299. Harper J, Skerry C, Davis SL, Tasneen R, Weir M, Kramnik I, et al. Mouse model of necrotic tuberculosis granulomas develops hypoxic lesions. *J Infect Dis.* 2012; 205(4):595–602. PubMed PMID: 22198962; PubMed Central PMCID: PMC3266133. [PubMed: 22198962]
300. Desai CR, Heera S, Patel A, Babrekar AB, Mahashur AA, Kamat SR. Role of metronidazole in improving response and specific drug sensitivity in advanced pulmonary tuberculosis. *The Journal of the Association of Physicians of India.* 1989; 37(11):694–697. PubMed PMID: 2632532. [PubMed: 2632532]
301. Lardner A. The effects of extracellular pH on immune function. *Journal of leukocyte biology.* 2001; 69(4):522–530. PubMed PMID: 11310837. [PubMed: 11310837]
302. Eoh H, Rhee KY. Methylcitrate cycle defines the bactericidal essentiality of isocitrate lyase for survival of *Mycobacterium tuberculosis* on fatty acids. *Proc Natl Acad Sci U S A.* 2014; 111(13): 4976–4981. PubMed PMID: 24639517; PubMed Central PMCID: PMC3977286. [PubMed: 24639517]
303. Dartois V, Barry CE 3rd. A medicinal chemists' guide to the unique difficulties of lead optimization for tuberculosis. *Bioorganic & medicinal chemistry letters.* 2013; 23(17):4741–4750. PubMed PMID: 23910985; PubMed Central PMCID: PMC3789655. [PubMed: 23910985]
304. Dannenberg AM Jr. Immunopathogenesis of pulmonary tuberculosis. *Hospital practice.* 1993; 28(1):51–58. PubMed PMID: 8419415.

305. Aly S, Wagner K, Keller C, Malm S, Malzan A, Brandau S, et al. Oxygen status of lung granulomas in *Mycobacterium tuberculosis*-infected mice. *The Journal of pathology*. 2006; 210(3):298–305. PubMed PMID: 17001607. [PubMed: 17001607]
306. Schon T, Elmberger G, Negesse Y, Pando RH, Sundqvist T, Britton S. Local production of nitric oxide in patients with tuberculosis. *Int J Tuberc Lung Dis*. 2004; 8(9):1134–1137. PubMed PMID: 15455601. [PubMed: 15455601]
307. Nicholson S, Bonecini-Almeida Mda G, Lapa e Silva JR, Nathan C, Xie QW, Mumford R, et al. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med*. 1996; 183(5):2293–2302. PubMed PMID: 8642338. [PubMed: 8642338]
308. Facchetti F, Vermi W, Fiorentini S, Chilosi M, Caruso A, Duse M, et al. Expression of inducible nitric oxide synthase in human granulomas and histiocytic reactions. *Am J Pathol*. 1999; 154(1): 145–152. PubMed PMID: 9916929. [PubMed: 9916929]
309. Choi HS, Rai PR, Chu HW, Cool C, Chan ED. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. *Am J Respir Crit Care Med*. 2002; 166(2):178–186. PubMed PMID: 12119230. [PubMed: 12119230]
310. Nathan C. Inducible nitric oxide synthase in the tuberculous human lung. *Am J Respir Crit Care Med*. 2002; 166(2):130–131. PubMed PMID: 12119220. [PubMed: 12119220]
311. Nathan C, Cunningham-Bussell A. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. *Nature reviews Immunology*. 2013; 13(5):349–361. PubMed PMID: 23618831; PubMed Central PMCID: PMC4250048.
312. Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A*. 2000; 97(16):8841–8848. PubMed PMID: 10922044; PubMed Central PMCID: PMC34021. [PubMed: 10922044]
313. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*. 2007; 130(5):797–810. PubMed PMID: 17803904. [PubMed: 17803904]
314. Ng VH, Cox JS, Sousa AO, MacMicking JD, McKinney JD. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. *Mol Microbiol*. 2004; 52(5):1291–1302. PubMed PMID: 15165233. [PubMed: 15165233]
315. Skaar EP. The battle for iron between bacterial pathogens and their vertebrate hosts. *PLoS pathogens*. 2010; 6(8):e1000949. PubMed PMID: 20711357; PubMed Central PMCID: PMC2920840. [PubMed: 20711357]
316. Goodsmith N, Guo XV, Vandal OH, Vaubourgeix J, Wang R, Botella H, et al. Disruption of an M. tuberculosis membrane protein causes a magnesium-dependent cell division defect and failure to persist in mice. *PLoS pathogens*. 2015; 11(2):e1004645. PubMed PMID: 25658098; PubMed Central PMCID: PMC4450064. [PubMed: 25658098]
317. Wolschendorf F, Ackart D, Shrestha TB, Hascall-Dove L, Nolan S, Lamichhane G, et al. Copper resistance is essential for virulence of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. 2010 Epub 2011/01/06. PubMed PMID: 21205886.
318. Rowland JL, Niederweis M. A multicopper oxidase is required for copper resistance in *Mycobacterium tuberculosis*. *J Bacteriol*. 2013; 195(16):3724–3733. PubMed PMID: 23772064; PubMed Central PMCID: PMC3754562. [PubMed: 23772064]
319. Rowland JL, Niederweis M. Resistance mechanisms of *Mycobacterium tuberculosis* against phagosomal copper overload. *Tuberculosis (Edinb)*. 2012; 92(3):202–210. PubMed PMID: 22361385; PubMed Central PMCID: PMC3323751. [PubMed: 22361385]
320. Darwin KH. *Mycobacterium tuberculosis* and Copper: A Newly Appreciated Defense against an Old Foe? *J Biol Chem*. 2015; 290(31):18962–18966. PubMed PMID: 26055711; PubMed Central PMCID: PMC4521017. [PubMed: 26055711]
321. White C, Lee J, Kambe T, Fritsche K, Petris MJ. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. *J Biol Chem*. 2009; 284(49):33949–33956. PubMed PMID: 19808669; PubMed Central PMCID: PMC2797165. [PubMed: 19808669]
322. Tan S, Sukumar N, Abramovitch RB, Parish T, Russell DG. *Mycobacterium tuberculosis* responds to chloride and pH as synergistic cues to the immune status of its host cell. *PLoS*

- pathogens. 2013; 9(4):e1003282. PubMed PMID: 23592993; PubMed Central PMCID: PMC3616970. [PubMed: 23592993]
323. Larrouy-Maumus G, Marino LB, Madduri AVR, Ragan TJ, Hunt DM, Bassano L, et al. Cell-Envelope Remodeling as a Determinant of Phenotypic Antibacterial Tolerance in *Mycobacterium tuberculosis*. ACS Infectious Diseases. 2016; 2(5):352–360. [PubMed: 27231718]
 324. Scott CC, Gruenberg J. Ion flux and the function of endosomes and lysosomes: pH is just the start: the flux of ions across endosomal membranes influences endosome function not only through regulation of the luminal pH. BioEssays : news and reviews in molecular, cellular and developmental biology. 2011; 33(2):103–110. PubMed PMID: 21140470.
 325. Jiang L, Salao K, Li H, Rybicka JM, Yates RM, Luo XW, et al. Intracellular chloride channel protein CLIC1 regulates macrophage function through modulation of phagosomal acidification. Journal of cell science. 2012; 125(Pt 22):5479–5488. PubMed PMID: 22956539; PubMed Central PMCID: PMC3561857. [PubMed: 22956539]
 326. McKinney JD, Honer zu Bentrup K, Munoz-Elias EJ, Miczak A, Chen B, Chan WT, et al. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature. 2000; 406(6797):735–738. Epub 2000/08/30. PubMed PMID: 10963599. [PubMed: 10963599]
 327. Munoz-Elias EJ, Upton AM, Cherian J, McKinney JD. Role of the methylcitrate cycle in *Mycobacterium tuberculosis* metabolism, intracellular growth, and virulence. Mol Microbiol. 2006; 60(5):1109–1122. Epub 2006/05/13. PubMed PMID: 16689789. [PubMed: 16689789]
 328. Beste DJ, Noh K, Niedenfuhr S, Mendum TA, Hawkins ND, Ward JL, et al. 13C-flux spectral analysis of host-pathogen metabolism reveals a mixed diet for intracellular *Mycobacterium tuberculosis*. Chem Biol. 2013; 20(8):1012–1021. PubMed PMID: 23911587; PubMed Central PMCID: PMC3752972. [PubMed: 23911587]
 329. Pandey AK, Sassetti CM. Mycobacterial persistence requires the utilization of host cholesterol. Proc Natl Acad Sci U S A. 2008; 105(11):4376–4380. PubMed PMID: 18334639; PubMed Central PMCID: PMC2393810. [PubMed: 18334639]
 330. Baek SH, Li AH, Sassetti CM. Metabolic regulation of mycobacterial growth and antibiotic sensitivity. PLoS biology. 2011; 9(5):e1001065. PubMed PMID: 21629732; PubMed Central PMCID: PMC3101192. [PubMed: 21629732]
 331. Shi L, Sohaskey CD, Pheiffer C, Datta P, Parks M, McFadden J, et al. Carbon flux rerouting during *Mycobacterium tuberculosis* growth arrest. Mol Microbiol. 2010; 78(5):1199–1215. PubMed PMID: 21091505; PubMed Central PMCID: PMC3072047. [PubMed: 21091505]
 332. Larsen MH, Biermann K, Chen B, Hsu T, Sambandamurthy VK, Lackner AA, et al. Efficacy and safety of live attenuated persistent and rapidly cleared *Mycobacterium tuberculosis* vaccine candidates in non-human primates. Vaccine. 2009; 27(34):4709–4717. PubMed PMID: 19500524; PubMed Central PMCID: PMC3512200. [PubMed: 19500524]
 333. Sambandamurthy VK, Derrick SC, Jalapathy KV, Chen B, Russell RG, Morris SL, et al. Long-term protection against tuberculosis following vaccination with a severely attenuated double lysine and pantothenate auxotroph of *Mycobacterium tuberculosis*. Infect Immun. 2005; 73(2): 1196–1203. PubMed PMID: 15664964; PubMed Central PMCID: PMC547051. [PubMed: 15664964]
 334. Gouzy A, Poquet Y, Neyrolles O. Nitrogen metabolism in *Mycobacterium tuberculosis* physiology and virulence. Nat Rev Microbiol. 2014; 12(11):729–737. PubMed PMID: 25244084. [PubMed: 25244084]
 335. Cunningham-Bussell A, Bange FC, Nathan CF. Nitrite impacts the survival of *Mycobacterium tuberculosis* in response to isoniazid and hydrogen peroxide. MicrobiologyOpen. 2013; 2(6):901–911. PubMed PMID: 24019302; PubMed Central PMCID: PMC3892337. [PubMed: 24019302]

Summary of key recommendations

- Numerous variables need to be considered when designing HTS against nonreplicating bacteria, including post-screening assays and how to test molecules in animal models of tuberculosis.
- When compounds are reported to kill nonreplicating, phenotypically tolerant mycobacteria, the conditions need to be specified and the assumption of generalizability to other conditions avoided. Whether the compounds kill in more than one model of nonreplication needs to be determined. The structure of the compounds under the test conditions needs to be verified. That the killing took place under nonreplicating conditions, rather than upon removal of the cells from those conditions, needs to be validated.
- When compounds kill bacteria under both replicating and nonreplicating conditions, it should not be assumed that the targets are the same in both cases. Conversely, it should not be assumed that lack of killing under nonreplicating conditions means that the target of a compound that is active in replicating conditions has become dispensable when the bacteria are no longer replicating.
- The field will experience substantial advances when we can test large numbers of nonreplicating-active molecules in diverse animal models and analyze and publish negative as well as positive results.

Figure 1. Strategies to evaluate the viability of nonreplicating mycobacteria for HTS. Arrow color indicates the quality of each readout strategy (considering robustness, ease of use, dynamic range, etc.) as excellent (**green arrows**), average to poor (**black arrows**), or infeasible (**red line**). Compound carry-over may result from compound transfer from the nonreplicating assay to replicating assay bacteriologic growth medium, or, by compound adherence to the bacterial cell wall.

Figure 2.

Selecting and designing nonreplicating models. **Left:** non-exhaustive list of models of class I and class II nonreplication. **Right:** variables to consider when designing models. **Center, bottom:** potential activity profiles of nonreplicating actives. The success of compounds targeting nonreplicating mycobacteria is dependent on the interactions among models, variables, and activity profiles. The term “DD Mtb” (differentially detectable M. tuberculosis) is used interchangeably with “viable-but-nonculturable” (VBNC) Mtb.

Figure 3.

Compound transformation during screening assays. **(a)** In cell-free, nonreplicating conditions imposed by the multi-stress model, oxyphenbutazone (**left**) rapidly transforms in acidic and nitrosative conditions to the intermediate, 4-hydroxy-oxyphenbutazone (**center**), which further transforms to 4-hydroxy-oxyphenbutazone quinoneimine (**right**). The electrophilic quinoneimine (**red**) can react at carbon atoms (**green**) with intrabacterial nucleophiles such as N-acetyl cysteine (NAC) and/or mycothiol (MSH). **(b)** Predicted, and

experimentally validated, points of compound modification that may occur during phenotypic screening.

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a.

in vitro	R	no
	NR	active
	macs	active
in vivo	mice	no
	humans	n.t.

b.

in vitro	R	active
	NR	active
	macs	active
in vivo	mice	active
	humans	active

c.

in vitro	R	no
	NR	active
	macs	active
in vivo	mice	active
	humans	no

in vitro	R	no *
	NR	active
	macs	unknown #
in vivo	mice	active
	humans	active

Figure 4.

Proof-of-concept molecules. Molecules with nonreplicating activity that serve as proof-of-concept include those that **(a)** selectively kill nonreplicating mycobacteria; **(b)** have dual activity, kill mycobacteria in the majority of nonreplicating models, and are effective at treating tuberculosis in animal models; and, **(c)** have selective activity against slowly replicating or nonreplicating mycobacteria and are efficacious in tuberculosis models. n.t. = Not tested.

* = Pyrazinamide has activity against slowly replicating mycobacteria.

= Experimental data indicates that pyrazinamide is inactive against intracellular mycobacteria *in vitro* (294, 295). However, pyrazinamide's dependency on an acidic environment for activity, and potent *in vivo* activity, suggests that it kills intracellular mycobacteria during animal and human tuberculosis.

Figure 5.

Canonical and non-canonical targets of dual-active molecules. Dual active molecules, which have bacteriostatic or bactericidal activity against replicating *M. tuberculosis* and bactericidal activity against nonreplicating *M. tuberculosis* are often presumed to engage the same target under both conditions. Dual active molecules may exert activity against nonreplicating mycobacteria via novel targets or non-specific mechanisms. The list of dual active molecules is not exhaustive.

a. lipid synthesis **b. DNA synthesis** **c. RNA synthesis**

d. protein synthesis **e. peptidoglycan synthesis** **Figure 6.**

Replicating and nonreplicating mycobacteria may share common targets. Examples of compounds that engage standard antibiotic target pathways under replicating conditions, and also kill nonreplicating mycobacteria, include inhibitors of the biosynthesis of (a) lipids, (b) DNA, (c) RNA, (d) protein, and (e) peptidoglycan.

quinolines

8-hydroxyquinolines

4-hydroxyquinolines

Figure 7.
Quinolines.

Figure 8.
Quinolones.

46
cyclomarin A

47
ecumicin

48
lassomycin

49
ADEP-4

50
GL-5

51

52
DPLG-2

Figure 9.
Compounds targeting the proteostasis and proteolysis pathways.

a. carbon starvation HTS **b. severe hypoxia HTS** **c. multistress HTS** **d. acidic pH HTS** **e. biofilm HTS** **Figure 10.**

Representative compounds identified by whole-cell HTS against mycobacteria rendered nonreplicating by (a) carbon-starvation (54); (b) hypoxia (29); (c) multiple stresses, including low pH, nitric oxide and reactive nitrogen intermediates, hypoxia, and a fatty acid carbon source (28, 53, 147); (d) acidic pH (119); and (e) culture as a biofilm (102).

Figure 11.
Nitro-containing compounds.

Figure 12.
Compounds that depolarize the mycobacterial membrane.

a. **b.** **Figure 13.**

Salicylanilides are protonophores. The commonly drawn structure of niclosamide is represented in **(a, left)**. Compound S-13, which was used for experimental logP calculations (268), is shown for reference **(a, right)**. As illustrated by niclosamide, salicylanilides capture protons by forming a stable pseudo-6-membered ring via hydrogen bonding **(b)**. Once inside the bacterial cell and releasing their proton, they maintain a stable anionic form from electron delocalization. Adapted from Terada (268).

98
auranofin

99

100

101

102

103

104

Figure 14.
Additional compounds that kill nonreplicating mycobacteria.

Table 1Evaluating the relationship between hypoxia and metronidazole activity *in vitro* and *in vivo*

Model	Method to measure or demonstrate hypoxia	Casating granulomas?	Activity of metronidazole	References
Wayne <i>in vitro</i> model of dormancy	< 0.06% O ₂ , methylene blue decolorization	No	Active	(19, 95)
Mice: C57Bl/6	Pimonidazole (immunohistochemistry)	No	Inactive	(13, 17, 296, 297)
Mouse: BALB/c	Not tested	No	Inactive	(298)
Mouse: C3HeB/FeJ "Kramnik model"	Copper(II)-diacetyl-bis(<i>N</i> ⁴ -methyl-thiosemicarbazone, pimonidazole (immunohistochemistry), gene expression of hypoxia-associated genes	Yes	Inactive	(64, 298, 299)
Guinea pig	Pimonidazole (immunohistochemistry)	Yes	Inactive	(15, 17)
Rabbit	Pimonidazole (immunohistochemistry), fiber-optic O ₂ probe	Yes	Active	(17)
Non-human primates	Pimonidazole (immunohistochemistry)	Yes	Active	(16, 17)
Human	EF5 / ELK3-51 antibody, HIF-1α (hypoxia inducible factor) (immunohistochemistry), [¹⁸ F]-fluoromisonidazole (positron emission tomography imaging)	Yes	Inactive	(14, 247, 296, 300)

Table 2

Conditions encountered by *M. tuberculosis* that may contribute to suboptimal replication rates or complete growth stasis. Individual conditions, or combinations of them, are anticipated to lead to phenotypic tolerance observed in animal models of tuberculosis and in human patients

Condition(s)	Chemical mediator(s), examples	Location or situation, example(s)	Responsible for microenvironmental condition	References
Acidic pH	H ⁺	<i>M. tuberculosis</i> -containing phagosome	Immune-stimulated macrophages	(233, 234)
		Lactate in extracellular fluid at inflammatory sites	Crowding of stromal and parenchymal cells by macrophages, monocytes, dendritic cells, lymphocytes, neutrophils leading to enhanced reliance on glycolysis	(301)
		Succinate	Secreted by <i>M. tuberculosis</i> under hypoxic conditions	(170, 302)
Hypoxia	Limiting O ₂	Necrotic granulomas	Poor vascularization of necrotic granuloma that has a surrounding rim composed of epithelioid macrophages, T-cells, B-cells, and neutrophils; phagosomes of human macrophages infected with wild-type <i>M. tuberculosis</i>	(17, 21, 303–305)
Reactive nitrogen intermediates	[•] NO, ONOO ⁻ , ONOOH, [•] NO ₂ , N ₂ O ₃ , N ₂ O ₅	Macrophage phagosomes	Human blood, human interstitial fluid, diet, enterosalivary nitrite cycle, inducible and constitutive nitric oxide synthases in macrophages, fibroblasts, vascular smooth muscle, endothelium, and bronchial epithelium, <i>M. tuberculosis</i> under hypoxic conditions or in human macrophages	(27, 234, 306–310)
Reactive oxygen intermediates	[•] O ₂ ⁻ , H ₂ O ₂ , ROOH, [•] OH, ¹ O ₂ , O ₃ , HOCl, HOBr, HOI	Macrophage phagosomes, antibiotics	Activated macrophages and polymorphonuclear leukocytes, the respiratory chain, catabolism (enzymatic and non-enzymatic), response of <i>M. tuberculosis</i> to antibiotics	(66, 311–314)
Metal deficiency	Iron	Animal models, humans	Iron sequestration by host iron-binding proteins such as lactoferrin	(231, 315)
	Magnesium	Macrophage phagosome, mycobacterial membrane lipids	NRAMP ion transporter	(245, 316)
Metal intoxication	Cu(I)	Macrophage phagosome, blood, lungs	Copper transporter ATP7A, copper importer CTR1, ceruloplasmin, NO-dependent release of copper from Cu(I)-protein complexes	(142, 232, 317–321)
Osmolarity	Chloride ion	Macrophage phagosome, airway surface liquid	Chloride channels, such as chloride channel protein 1 (CLIC1)	(322–325)
Carbon sources that support slow growth or survival; growth rate less than maximal	Fatty acids (short/long chain; saturated/unsaturated), cholesterol, amino acids, CO ₂	Macrophage phagosomes, caseum, granulomas, adipose tissue	Krebs cycle, glycolysis	(235, 238, 239, 326–330)

Condition(s)	Chemical mediator(s), examples	Location or situation, example(s)	Responsible for microenvironmental condition	References
Nutritional starvation	Use of <i>M. tuberculosis</i> metabolic reserves: trehalose, glycogen, fatty acids, glutamate	Unlikely to occur	Krebs cycle, glyoxylate shunt	(12, 218, 331)
Amino acid or vitamin deficiency	Methionine, lysine, leucine, pantothenate	Macrophage phagosomes	Limited quantities <i>in vivo</i> , or amino acids and vitamins are not in a form accessible for mycobacterial uptake	(240–242, 332, 333)
Nitrogen metabolism	Amino acids, inorganic NH_4^+ , urea NO_3^- (nitrate)	Hypoxic lesions, and/or hypoxic-acidic phagosomes	<i>M. tuberculosis</i> respiring nitrate as an alternative electron acceptor, acid resistance, nutrient starvation	(21, 334, 335)

Table 3

Post-screening assays for molecules active on replicating and/or nonreplicating *M. tuberculosis*

Hit triage assay / study	Description and/or techniques used	Function
Validate chemical identity and purity	LC-MS, NMR	Sometimes overlooked, this is an essential component of HTS that can prevent heartbreak after years of work. It is imperative to confirm that the proposed structure is the molecule responsible for the observed activity.
Confirm activity with re-supplied compound; confirm activity with re-synthesized compound	Re-order from trusted supplier and conduct <i>de novo</i> chemical synthesis	
Medicinal chemist analysis for structural alerts or toxicophores	Organic, physical, and medicinal chemistry; cheminformatics; experience	Identification of structural alerts or toxicophores may predict alternative mechanisms of action (often general reactivity) and <i>in vivo</i> liabilities, such as liver toxicity or metabolism.
Glutathione reactivity	Mix compound with reduced glutathione and look for possible GSH-compound adducts	Highly unstable transformation products may be difficult to observe due to their transient nature. Glutathione, with a cysteine nucleophile, reacts avidly with electrophilic molecules and can form stable GSH-compound adducts.
Toxicity to HepG2 and/or Vero cells	EC ₅₀ determination by correlating ATP levels with cellular viability.	Both the human hepatocellular carcinoma cell lines (HepG2) and monkey kidney epithelial cell lines (Vero) are used to test for cellular toxicity; HepG2 cells also assay for possible bioactivation in the liver.
Determine the selectivity index (SI)	EC ₅₀ in Vero or HepG2 cells divided by the MIC ₉₀ against <i>M. tuberculosis</i>	Helps prioritize compounds that are more potent against <i>M. tuberculosis</i> than against eukaryotic cells. Typically, an SI of >10 is used as a threshold for SAR analysis, and an SI of >50 is required to advance to lead candidacy. The duration of compound exposure to <i>M. tuberculosis</i> and HepG2 / Vero cells and equivalence of serum proteins in the two assays should be taken into account.
Serum shift	Determine MIC ₉₀ in screening medium +/- 10% heat inactivated mouse or human serum.	A serum shift may indicate if a compound is highly protein bound and provide a warning that eukaryotic toxicity results, typically performed with 10% serum, may have underestimated toxicity.
Inoculum effect	Determine MIC ₉₀ at an OD ₅₈₀ of 0.01 and 0.10.	Some compounds such as beta-lactams are well known for inoculum effects, in which their potency decreases as the inoculum increases.
Microbiologic spectrum	Test activity against representative Gram-positive and Gram-negative bacteria, and fungi.	To determine if a compound has broad- or narrow-spectrum activity.
Frequency of resistance (FOR)	Plate 10 ⁶ to 10 ⁹ <i>M. tuberculosis</i> on agar plates containing 1-20X	Determine the number of pre-existing mutants in a population that are naturally resistant to a

Hit triage assay / study	Description and/or techniques used	Function
	the broth MIC ₉₀ of compound.	drug, or, determine if the drug itself is mutagenic. Acceptable FOR's are 1×10^{-6} (the FOR of isoniazid).
Test activity against drug resistant <i>M. tuberculosis</i> strains	Determine the MIC ₉₀ of a drug against <i>M. tuberculosis</i> strains resistant to isoniazid, rifampicin, streptomycin, ethionamide, ethambutol, etc.	A new agent should have activity against drug-resistant strains.
Test activity against clinical isolates of <i>M. tuberculosis</i>	Determine the MIC ₉₀ of a drug against clinical isolates from tuberculosis patients.	A new agent should have activity against strains of <i>M. tuberculosis</i> with diverse genotypes.
Genotoxicity	Ames and micronucleus.	Compound series with genotoxicity may be mutagenic. SAR campaigns may find analogues that are not mutagenic; if not, series should be deprioritized or terminated.
Activity against intracellular <i>M. tuberculosis</i>	Examples include <i>M. tuberculosis</i> infecting human blood monocytes, human macrophage cell lines such as THP-1, or murine J774 and RAW macrophage cell lines.	This assay usually omits immunological stimulation of the macrophages. Consequently <i>M. tuberculosis</i> is predominantly replicating.
Activity against <i>M. tuberculosis</i> grown with different carbon sources	Test acetate, propionate, dextrose, glutamate, glycerol (without dextrose)	It is essential to confirm that the activity of a molecule is not strictly dependent on the carbon source.
CFU assays: time- and dose-dependency	Test for CFU reduction at multiples of MIC ₉₀ and at different times of compound exposure.	The CFU assay is considered the gold standard to enumerate viable bacteria. Molecules that fail to demonstrate dose- and time-dependent kill may have a non-specific mechanism of action.

Table 4

Post-screening assays specific for nonreplicating active, or candidate dual-active molecules (active on both replicating- and nonreplicating-bacilli). These assays are used in addition to the standard post-screening assays (Table 3)

Assay / Method	Function / description	References
CARA (charcoal agar resazurin assay)	1) To distinguish replicating-, nonreplicating-, and dual-active molecules; 2) to distinguish replicating bacteriostatic and replicating bactericidal; 3) to serve as a semi-quantitative pre-test prior to CFU assays; 4) to permit testing the impact of a compound over a wide range of concentrations and time points.	(30, 147)
Cell-free stability assay	To determine if a compound undergoes structural modification in the cell-free medium used for the nonreplicating HTS model.	(28, 53)
Pre-NR assay	To determine if any potential transformation products have activity against replicating <i>M. tuberculosis</i> . Compounds are pre-incubated for ~ 24 hours in cell-free nonreplicating medium, and then added to replicating culture of <i>M. tuberculosis</i> to determine if any potential transformation products have bacteriostatic or bactericidal activity.	(28, 53)
Membrane depolarization assays: membrane potential (ψ) and transmembrane proton concentration gradient (pH)	To identify if the mechanism of action is related to inhibition of an enzymatic target or non-specific depolarization of the bacterial membrane.	(159, 260)
Intrabacterial pH	To determine if a compound impacts the ability of <i>M. tuberculosis</i> to regulate intrabacterial pH. Compounds active in acidic nonreplicating models often function as protonophores and decrease intrabacterial pH. This assay employs a pH-sensitive GFP variant.	(119, 120, 123, 125)
Activity against intracellular <i>M. tuberculosis</i> IFN γ -activated, bone-marrow derived murine macrophages	To determine if a compound has bactericidal activity against intracellular <i>M. tuberculosis</i> . Immunological stimulation of macrophages adapts the <i>M. tuberculosis</i> -containing phagosome to a microenvironment that is no longer conducive for exponential replication. Such conditions include acidic pH, a shift to using fatty acid carbon sources, mild hypoxia, reactive oxygen and nitrogen intermediates, itaconic acid production, metal starvation, metal intoxication, etc.	(49, 158, 233, 234)
CFU-based time-kill curves under both replicating and nonreplicating conditions	To enumerate viable <i>M. tuberculosis</i> after exposure to a test agent. The CFU assay is widely considered the gold standard to experimentally demonstrate a compound's activity on replicating and nonreplicating bacteria.	(28, 34, 53, 100)
Inclusion of 0.4% (w/v) activated charcoal or 5% BSA (w/v) in bacteriologic agar plates used to enumerate CFU assays	To avoid carry-over effects when enumerating viable <i>M. tuberculosis</i> by a CFU assay. Dual active molecules, and potent replicating active molecules, may artifactually display activity due to compound carry-over in agar plates. Activated charcoal or BSA can sequester carry-over compound. Activated charcoal is preferred due to its ability to bind a wider range of compounds.	(30–33)
Deconvolution of nonreplicating assay conditions	To determine which stress, or combination of stresses, is required for a compound to kill nonreplicating <i>M. tuberculosis</i> .	(28, 53)
Test activity in alternative class I and/or class II models	To identify candidate molecules with pan-activity against nonreplicating mycobacteria.	(50, 51, 54, 100)
Determination of the Wayne Cidal Concentration (WCC ₉₀) and Loebel Cidal Concentration	To determine the concentration of compound that leads to killing 90% of the starting inoculum under hypoxic (Wayne) or nutrient starvation (Loebel)	(51, 159)

Assay / Method	Function / description	References
(LCC ₉₀)	conditions.	
Test for activity against nonreplicating Gram-positive and/or Gram-negative bacteria	To determine if a compound kills non-mycobacterial bacterial species under nonreplicating conditions. This is an adaptation of the standard “microbial spectrum” typically run under replicating conditions.	(28)
Most probable number assay (MPN)	To quantitate “viable-but-not-culturable” (VBNC), otherwise known as “differentially detectable” <i>M. tuberculosis</i> (DD Mtb), that fail to grow on standard agar-based bacteriologic media. The ability to kill DD Mtb is considered a highly desirable property of compounds targeting nonreplicating persisters.	(10)