Circumventing Drug Resistance with Small Molecule Inhibitors of Mycobacterium tuberculosis

Gregory Alexander Harrison

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Circumventing Drug Resistance with Small Molecule Inhibitors of *Mycobacterium tuberculosis*

by

Gregory A. Harrison

A dissertation presented to
Washington University in St. Louis
in partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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With Gratitude,

Gregory A. Harrison

Washington University in St. Louis

May 2023
Dedicated to my wonderful family
ABSTRACT OF THE DISSERTATION

Circumventing Drug Resistance with Small Molecule Inhibitors of *Mycobacterium tuberculosis*

by

Gregory A. Harrison

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Microbiology and Microbial Pathogenesis

Washington University in St. Louis, 2023

Professor Christina L. Stallings, Chair

The disease tuberculosis caused by *Mycobacterium tuberculosis* (*Mtb*) remains a global health threat, and the antibiotic resistance crisis challenges our ability to treat *Mtb* infections. For example, out of the approximately 10 million cases of tuberculosis each year, an estimated 10.7% of new cases and 27.2% of previously treated cases are resistant to the frontline antibiotic isoniazid (INH). There is a clear need for new therapies that enhance our current regimen and target these genetically resistant strains.

Identifying ways to enhance the antimicrobial activity of INH has the potential to significantly improve the efficacy of frontline treatment regimens. To this end, our lab identified a small molecule potentiator of INH, C10. While C10 on its own has poor growth-inhibitory activity, this compound enhances the bactericidal activity of INH against *Mtb*. Remarkably, we discovered that C10 suppresses the emergence of INH-resistant mutants *in vitro* and can even re-sensitize some INH-resistant mutants to this antibiotic. INH is a cell-wall targeting antibiotic that inhibits the biosynthesis of mycolic acid, an essential lipid in the outer layer of the mycobacterial cell envelope. However, INH is a pro-drug that must first be converted to its active form INH-NAD through the activity of the bacterial enzyme KatG. Activated INH-NAD can then bind and
inhibit its target, the essential mycolic acid biosynthesis enzyme InhA. The vast majority of INH resistant isolates harbor mutations that decrease the activity of the activating enzyme KatG. Our work demonstrated that C10 could re-sensitize a set of INH-resistant katG mutants to INH, revealing that INH resistance is not absolute and can be reversed.

In pursuit of the mechanism of action of C10, I used transcriptional profiling, forward genetics, and assays of bacterial physiology to demonstrate that C10 disrupts Mtb energy metabolism, leading to decreased respiration and depletion of bacterial ATP. We found that at very high concentrations (>50μM), C10 exhibited bacterial toxicity that was mediated through disruption of Mtb energy metabolism. However, using a combination of genetic and chemical biology approaches, we discovered that the ability of C10 to disrupt Mtb energy metabolism was neither required nor sufficient to potentiate the bactericidal activity of INH. C10 must impart some other effect on Mtb that elicits the increased INH sensitivity.

To more specifically understand how C10 potentiates INH, we selected for mutants resistant to both INH and C10. The INH/C10-resistant mutants harbored katG-null mutations that abolished synthesis of INH-NAD, rendering the strains resistant to INH even in the presence of C10. In contrast, only INH-resistant strains that retained residual KatG activity and accumulated a low level of INH-NAD could be re-sensitized to inhibition by C10. Therefore, some low level of KatG activity is required for C10 to potentiate INH.

The best studied mechanism to enhance INH activity involves increasing the amount of INH-NAD synthesis in the bacteria. However, through a series of assays, we found that C10 sensitized Mtb to killing by INH without increasing KatG activity or INH-NAD levels. C10 therefore sensitizes Mtb to INH-NAD after it is synthesized without changing its levels, such that resistant mutants that accumulate sub-inhibitory levels of INH-NAD become inhibited by this
concentration of INH-NAD in the presence of C10. In support of this model, we discovered that C10 sensitizes the bacteria to killing by the direct InhA inhibitor NITD-916. Notably, decreasing InhA expression or activity could sensitize *Mtb* to InhA inhibitors by titrating the concentration of antibiotic required to inhibit this target. However, we showed that C10 on its own does not decrease InhA expression or activity. Therefore, C10 enhances the bacterial vulnerability to InhA inhibitors through an un-precedented mechanism. These findings have implications for mycolic acid targeting antibiotics currently in use in the clinic and in clinical development.

The target and precise mechanism of action of C10 remains unknown. However, by using chemical genetic approaches to interrogate the pleiotropic effects of C10 against *Mtb*, my thesis work has enhanced our understanding of *Mtb* physiology and uncovered a novel mechanism to circumvent *Mtb* drug resistance.

In an independent approach to identify compounds that inhibit *Mtb* through a novel mechanism of action, our lab screened a small library of nucleotide mimetics to identify inhibitors of *Mtb* growth. We discovered a new series of antmycobacterial compounds, 4-amino-thieno[2,3-d]pyrimidines, that potently inhibit the growth of *Mtb*. Using forward genetics and assays of *Mtb* bioenergetics, we found that 4-amino-thieno[2,3-d]pyrimidines inhibit the QcrB subunit of the electron transport chain enzyme cytochrome *bc*1, a validated drug target.

Collectively this work highlights that *Mtb* energy metabolism is a promising target for the development of novel small molecule *Mtb* inhibitors. In attempts to address the *Mtb* drug resistance crisis, I have contributed to our understanding of how C10 sensitizes *Mtb* to clinically relevant antibiotics, and I have described a new chemical class of respiration inhibitors, 4-amino-thieno[2,3-d]pyrimidines, which inhibit a known *Mtb* drug target.
Chapter 1: Introduction

Some parts of this introduction in Sections 1.1.1, 1.1.2, and 1.1.3 contain passages originally published as part of a review article - Samuels, Wang, & Harrison, et al 2022 Frontiers in Cellular and Infection Microbiology 1 (PMCID: PMC9441742) with the following co-contributors:

Amanda N. Samuels#, Erin R. Wang#, Gregory A. Harrison#, Joy C. Valenta, and Christina L. Stallings*

#ANS, ERW, and GAH contributed equally to this work and are co-first authors.

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The remainder of this chapter is unpublished and was written by GAH as the sole author
1.1 **Tuberculosis is a global health threat**

1.1.1 **Tuberculosis infections are recalcitrant to antibiotic therapy**

Tuberculosis, an infection caused by the pathogen *Mycobacterium tuberculosis* (*Mtb*), is one of the leading causes of death world-wide by an infectious agent. The standard treatment regimen for drug sensitive *Mtb* infections lasts 6 months, consisting of a 2-month intensive phase with daily doses of isoniazid (INH), ethambutol (EMB), rifampicin (RIF), and pyrazinamide (PZA), followed by a continuation phase of at least 4 months of INH and RIF. In 2022, the World Health Organization approved the recommendation for a shortened 4-month treatment regimen that can be made available to some patients, which includes a 2-month intensive phase of INH, rifapentine (RPT), moxifloxacin (MXF), and PZA followed by 2 months and 1 week of INH, rifapentine, and moxifloxacin. Since both the longer 6-month and the shorter 4-month regimens rely heavily on the antibiotic INH for both intensive and continuation phases of treatment, INH is an indispensable frontline drug for antituberculosis therapy. However, the antibiotic resistance crisis makes treating these difficult infections even more challenging. Antibiotic resistant *Mtb* is a global health threat. For example, an estimated 10.7% of newly infected and 27.2% of previously treated cases are resistant to INH each year.

In addition, treatment failure and relapse can occur even in the absence of drug resistance. Dating as far back as the 1950’s, it is documented that *Mtb* can be recovered from some patients after antibiotic treatment, with a fraction of these isolates remaining drug sensitive in vitro. In a 2014 study, 8% of patients that were treated with the standard of care had an unfavorable outcome, the most common of which was infection relapse. Shortening the antibiotic regimen resulted in even further increased rates of treatment failure and relapse. In this study, only 25% of the
patients receiving the standard of care that experienced infection relapse were suspected of acquiring drug resistance. Another study collected serial *Mtb* isolates from tuberculosis patients that had relapsed infection after antibiotic treatment, where relapse was defined by paired isolates exhibiting 0-6 single nucleotide polymorphisms by whole genome sequencing. In this study, all the relapsed *Mtb* isolates were drug sensitive *in vitro*. Collectively, these data support that a reservoir of drug sensitive *Mtb* can persist in the host despite antibiotic therapy, contributing to treatment failure in some patients, even in the highly controlled setting of a clinical trial.

### 1.1.2 Factors that contribute to *Mtb* surviving antibiotic treatment

Multiple factors have been identified that enable *Mtb* to persist in the host during antibiotic treatment without acquiring a drug resistance mutation. One factor is the pathology that develops within the lung during *Mtb* infection. During infection, the interaction between *Mtb* and the host immune response can result in the development of a granuloma, which is made up of host immune cells, *Mtb*, and tissue debris. Antibiotic penetration into the granuloma can be limited based on the chemical properties of the antibiotic, which creates an unequal distribution of the antibiotic across the various sites where *Mtb* resides.

In addition to the host response impacting antibiotic accessibility to *Mtb*, the pathogen itself changes its physiology in response to the host environment, resulting in phenotypic drug tolerance. Importantly, drug tolerance is different from drug resistance in that a drug tolerant population can survive in the presence of an antibiotic but cannot grow until the antibiotic pressure is removed, whereas a drug resistant population can both survive and replicate in the presence of an antibiotic. In unstressed axenic culture conditions, *Mtb* populations display a basal level of heterogeneity such that a subpopulation of bacteria is transiently tolerant to antibiotics. Because of this drug tolerant subpopulation, treatment with a bactericidal antibiotic, such as INH or RIF, leads to a
significant decrease in viable bacteria, but fails to sterilize the culture \(^{17-19}\). Asymmetric cell division contributes to heterogeneity in the bacterial population, driving the formation of drug tolerant sub-populations of bacteria within the community \(^{15,16}\). In addition, there are stochastic differences in gene expression within mycobacterial cultures that can affect antibiotic susceptibility. For example, mycobacteria exhibit stochastic variation in the expression of KatG, which is required to activate the pro-drug INH, leading to a small population of bacteria with transiently low KatG expression that can survive exposure to INH \(^{20}\).

The proportion of drug tolerant \(Mtb\) is higher \textit{in vivo} when compared to the small population that exists at basal levels in unstressed axenic cultures. \(Mtb\) directly isolated from patient sputum samples exhibited a nearly 10-fold reduction in killing by streptomycin, INH, EMB, or Rif in comparison to when those same isolates were passaged through normal culture conditions \(^{21}\). \(Mtb\) in caseum isolated from infected rabbit granulomas also exhibited a >100-fold increase in the minimum bactericidal concentration for Rif and INH compared to \(Mtb\) growing \textit{in vitro} \(^{22}\). Therefore, the \(Mtb\) population at the site of infection is enriched for drug tolerant cells, indicating that the host environment causes the \(Mtb\) population to shift towards a more drug tolerant state.

\subsection*{1.1.3 Stresses encountered in the host promote drug tolerance}

During intracellular infection of macrophages, \(Mtb\) is exposed to low pH, nitrosative stress, oxidative stress, osmotic changes, carbohydrate limitation, and cell envelope damage \(^{23-26}\). The environment within granulomas also poses additional stresses on \(Mtb\), where granulomas can be hypoxic \(^{27}\), contain host factors that sequester iron \(^{28}\), and harbor host enzymes that produce reactive oxygen species \(^{29}\). Despite this harsh host environment, \(Mtb\) can survive due to its robust stress response capabilities. \(Mtb\) responds transcriptionally and metabolically to survive exposure
to hypoxia, nitric oxide, reactive oxygen species, carbon limitation, iron limitation, and low pH. Importantly, when exposed to stress in vitro, such as hypoxia, low pH, changes in osmolarity, or nutrient limitation, the proportion of drug tolerant Mycobacterium tuberculosis (Mtb) increases, leading to higher minimal inhibitory concentrations or minimum bactericidal concentrations for several antibiotics. These data support that exposure to host derived stresses contributes to the increased Mtb antibiotic tolerance observed during infection.

1.1.4 There is a dire need to identify ways to target drug tolerant and drug resistant Mtb

The heterogeneity in the susceptibility of the bacterial population as well as the increased proportion of drug tolerant Mtb that exist in vivo make it necessary to treat these infections over the course of several months with daily antibiotics to effectively clear the infection. However, this long duration of treatment causes Mtb to be exposed to fluctuating concentrations of antibiotics over several months. Furthermore, there can be lapses in treatment adherence due to multiple factors including toxic side effects from long-term antibiotic use and access to these expensive drugs, facilitating the emergence of drug resistant mutant isolates that arise spontaneously during infection. Serial whole genome sequencing of Mtb from patients during the course of infection indicates that antibiotic therapy enriches for mutants that acquire drug resistance mutations over time, suggesting that the long treatment duration actually selects for these drug resistant isolates. Consistent with these studies, the rate of drug resistance in previously treated cases is more than twice the rate of drug resistance in newly acquired infections, supporting that treatment for tuberculosis is a risk factor for the acquisition of a resistant infection. One approach to curtail the emergence of drug resistant isolates is to develop therapeutics that can target drug tolerant populations of Mtb. Enhancing the potency of the current frontline antibiotics against these drug
tolerant bacteria could lead to more effective frontline regimens and shorter treatment durations, which could decrease the opportunities for drug resistant mutants to emerge.

To this end, previous members of the Stallings lab screened for small molecules that would enhance the efficacy of antibiotics against drug tolerant *Mtb* cultured in hypoxic conditions. Through this screen, we identified a compound, C10, which enhanced the bactericidal activity of INH even against these drug tolerant, hypoxic bacteria \(^{45}\). The discovery and characterization of C10 is described in further detail in Chapters 2-4. Notably, C10 did not potentiate the activity of RIF, EMB, or streptomycin in these conditions, indicating that the compound has a unique impact on the bactericidal effect of INH \(^{45}\). Since INH is a cornerstone drug that is an essential component of our frontline antibiotic regimens, C10 represents a promising approach to better target these drug tolerant bacteria with this antibiotic. These findings prompted me to become particularly interested in INH, and to seek to understand how C10 might potentiate its activity.

### 1.2 INH discovery and mechanism of action

#### 1.2.1 Discovery of INH

The discovery of INH was facilitated by the serendipitous finding in 1945 that the vitamin nicotinamide, provided to cancer patients in an attempt to alleviate the symptoms of radiation therapy, actually cleared tuberculosis lesions \(^{46,47}\). In the same year, it was found that supplementing *Mtb*-infected guinea pigs with extremely high amounts of nicotinamide prolonged their survival \(^{47,48}\). Inspired by the potential antitubercular activity of this vitamin supplement, multiple groups screened nicotinamide analogs in an attempt to identify a compound with more potent activity, and the anti-mycobacterial activity of INH was ultimately reported by multiple groups in 1952 \(^{49-53}\). The mechanism of action of INH was controversial for decades. However,
radioactive $^{14}$C-labeling of INH-treated *Mtb* in the 1970’s led to the discovery that INH inhibited the synthesis of mycolic acids, which are large lipid molecules that are an essential component of the mycobacterial cell wall $^{54-56}$. It would be decades before its precise mechanism was elucidated.

### 1.2.2 Mycobacterial cell wall overview

Mycobacteria belong to the phylum Actinobacteria, which is primarily comprised of Gram-positive organisms. However, the mycobacterial cell wall differs significantly from both Gram-positive and Gram-negative organisms. In mycobacteria, the cell wall is comprised of three distinct layers that are covalently linked to each other to form a structure called the mycolyl-arabinogalactan-peptidoglycan complex. The base of this complex is a thick layer of peptidoglycan, a polymer of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) amino-sugars with oligopeptide side-chains that can be crosslinked to form a mesh-like structure that encases the bacterial cell. The peptidoglycan layer is crosslinked to a long, branched polysaccharide termed arabinogalactan. The base of arabinogalactan consists of a 30-residue galactose chain attached to the MurNAc residues of peptidoglycan through a GlcNAc-L-rhamnose linker unit $^{57-61}$. Attached to residues 8, 10, and 12 of the galactan chain are long, branched arabinose sidechains $^{62,63}$. At the end of the arabinogalactan, the terminal arabinose residues are covalently linked to mycolic acids, forming the outermost layer of the mycolyl-arabinogalactan-peptidoglycan complex $^{64}$. This mycolic acid layer includes an outer leaflet of non-covalently attached, free lipids, including free mycolic acids and other lipids that are intercalated within this inner leaflet, together forming a thick, waxy layer that is critical for the integrity of the mycobacterial cell wall $^{65-67}$.

### 1.2.3 Mycolic acid biosynthesis

Mycolic acids are large fatty acids, with a unique structure comprising a long (C54-C63)
meromycolyl fatty acid chain that has an alkyl side chain (C22-C24) branching off the α carbon, and a hydroxyl group on the β carbon. The synthesis of these unusually large lipids requires a complicated, multi-step process, including a eukaryotic-like fatty acid synthase I (FAS-I), a multi-subunit fatty acid synthase II (FAS-II), and a series of additional enzymes.

The precursors of mycolic acids are synthesized by FAS-I. The mycobacterial FAS-I enzyme has a unique bimodal activity resulting in synthesis of both C16-C18 and C24-C26 fatty acids. The shorter C16-C18 fatty acid-CoA products can be synthesized by FAS-I de novo, but the enzyme has been shown to re-prime synthesis and elongate the C16-C18 fatty acids to C24-C26 fatty acid-CoA products. This bimodal activity of FAS-I is important because both shorter (C16-C18) and longer (C24-26) fatty acids are essential precursors of mycolic acid biosynthesis.

FAS-II further elongates these FAS-I products to form the long meromycolyl chain that becomes a part of mycolic acids. Unlike FAS-I, the FAS-II system is comprised of multiple distinct enzymes and is unable to synthesize fatty acids de novo, instead requiring the fatty acid-CoA products of FAS-I to prime fatty acid synthesis. FabH is the first enzyme in the FAS-II system and provides an essential link between the FAS-I and FAS-II systems. FabH is a β-ketoacyl-acyl carrier protein (ACP) synthase, which condenses a fatty acid-CoA substrate with a malonyl group that has been pre-loaded onto the ACP protein AcpM to generate β-ketoacyl-AcpM. Based on biochemical and structural data, the active site of FabH can only accommodate fatty acid substrates C16 or shorter, indicating that FAS-II relies on the shorter class of FAS-I products to prime synthesis. After loading by FabH, the AcpM protein then shuttles the growing lipid chain to the remaining FAS-II enzymes in a cycle of keto-reduction, dehydration, and enoyl-reduction. The NADPH-dependent keto-reduction is catalyzed by FabG1, generating a β-hydroxyacyl product. Next, HadB, catalyzes the removal of the hydroxyl group generating enoyl-AcpM.
AcpM is finally reduced by the NADH-dependent enoyl-ACP reductase InhA, converting the double bond to a saturated acyl chain that is ultimately 2 carbons longer than the starting acyl-CoA that was originally bound by FabH.

Once the acyl-AcpM is elongated by a single round of FAS-II synthesis, it is no longer a substrate for FabH, and requires a new class of β-ketoacyl-ACP synthase to re-initiate the cycle. Instead, either KasA or KasB re-initiates the FAS-II cycle by condensing the nascent acyl-AcpM with malonyl-AcpM, thereby adding an additional 2-carbon unit. Through multiple rounds of synthesis, FAS-II converts the shorter ~C16 FAS-I products into fatty acids longer than 50C. To complete the synthesis of the meromycolate chain, this long fatty acid must be modified. Two unsaturation points are introduced that cause the formation of kinks in the chain that allow the long chain to fold along itself, ultimately facilitating packing of these lipids in the cell envelope. In Mtb these unsaturation points are typically cyclopropyl, methoxy, keto, or hydroxyl groups. There are 8 S-adenosylmethionine (SAM)-dependent methyl transferases that are necessary to generate these modifications in Mtb. Notably, it is possible to delete all 8 of these enzymes in Mtb, suggesting that these modifications are dispensable for the survival of Mtb in culture. Yet the mutant strain lacking all 8 enzymes was attenuated in mice, indicating that these modifications are important for establishing an infection in the host. Once modified, the meromycolyl-AcpM is complete.

Next, the fatty acyl-AMP ligase FadD32 transfers the meromycolate group from AcpM to ATP, generating a meromycolyl-AMP intermediate, and then ligates this meromycolyl group into the ACP domain of the enzyme Pks13. In parallel, an enzyme complex formed by AccD4 and AccA3 carboxylate a second fatty acid-CoA substrate, which is to become the α-branch of the mycolic acid, to generate carboxyacyl-CoA and then ligate the carboxyacyl group into a second ACP domain of Pks13. AccA3/AccD4 exhibits a strong preference for C24-C26 fatty acid-
CoA substrates, suggesting that this enzyme requires the longer C24-C26 fatty acids derived from FAS-I. Pks13 then condenses the meromycolyl and carboxyacyl chains, forming a $\alpha$-alkyl, $\beta$-ketoacyl product. Nearly complete, Pks13 then transfers this product to a trehalose molecule, generating $\alpha$-alkyl, $\beta$-ketoacyl trehalose. The $\beta$-ketone group is ultimately reduced by the enzyme CmrA to a hydroxyl, in the final step of mycolic acid synthesis, thereby completing the formation of the mycolic acid species trehalose monomycolate (TMM).

The TMM is transported out the cell by the transporter MmpL3. Outside the cell the mycolyltransferases Ag85A, Ag85B, and Ag85C transfer the mycolic acid moiety from TMM to either the terminal arabinose residues of the arabinogalactan or to the trehalose residue of another TMM molecule forming trehalose dimycolate (TDM). The mycolic acids covalently attached to the arabinogalactan form the inner leaflet of the outer layer of the mycobacterial envelope. Free lipids such as TMM and TDM as well as a myriad of structurally diverse lipids are intercalated within the covalently attached mycolic acids, forming the waxy, nonpolar outer layer of the cell envelope.

1.2.4 The mechanism of action of INH – inhibition of mycolic acid biosynthesis

The mechanism of action of INH was not clearly resolved until the turn of the 21st century, nearly 50 years after its discovery. Early studies in 1954 by Middlebrook and colleagues characterizing INH-resistant mutants pointed to bacterial factors required for INH activity. While the lack of genetic data at this time precluded the identification of the exact mechanism of resistance in these strains, they observed that a predominant number of INH-resistant isolates were defective for catalase activity. It was later demonstrated that the gene katG, which encodes the sole bifunctional catalase-peroxidase enzyme in Mtb was required for bacterial susceptibility to INH, and mutations that abrogate the expression or activity of katG confers INH-resistance.
Indeed, mutation of katG is the predominant mechanism of INH-resistance observed in the clinic, accounting for over 90% of INH-resistant clinical isolates in some parts of the world. However, since it is dispensable for Mtb growth, KatG was unlikely to be the target of INH, leaving open the question of how INH inhibits Mtb growth.

Through a series of forward genetic approaches to isolate additional INH-resistant mutants in M. smegmatis, a S94A mutant allele in the gene inhA was identified in a subset of resistant isolates. It was shown that over-expression of inhA\textsuperscript{WT} or inhA\textsuperscript{S94A} was sufficient to confer INH resistance in Mtb. Additionally, engineering the S94A mutation into the endogenous inhA locus in the Mtb genome conferred INH resistance and abrogated the ability of INH to block mycolic acid biosynthesis, indicating that InhA is likely the physiologically relevant target of INH. Given the role of InhA in the FAS-II system, this model was consistent with the ability of INH treatment to block de novo mycolic acid biosynthesis in radiolabeling experiments. Furthermore, conditional inactivation of inhA in M. smegmatis was found to lead to accumulation of FAS-I products and bacterial cell lysis, similar to INH treatment.

However, INH failed to inhibit InhA activity directly. Using in vitro assays, it was demonstrated that INH must be activated before it can inhibit InhA. In a seminal discovery, the crystal structure of InhA was resolved in its inhibited form, revealing that InhA was bound to an INH-NAD adduct. It was subsequently found that KatG is required in Mtb to chemically modify INH, ultimately coupling INH to NAD(H) to generate this active INH-NAD adduct. As a catalase-peroxidase enzyme, the typical substrates of KatG are H$_2$O$_2$ and other peroxides. However, KatG acts on INH as a non-canonical substrate, generating an isonicotinyl radical that can covalently adduct onto various molecules within the cell, including the abundant cofactor NAD(H), generating INH-NAD. In follow-up studies, INH-NAD was shown directly to
inhibit InhA\textsuperscript{113,116}, and using mass spectrometry to directly monitor INH-NAD production in live \textit{Mtb}, it was found that INH-NAD is produced within \textit{Mtb} in a KatG-dependent manner\textsuperscript{117}. Together, these studies demonstrate that INH is activated by KatG to INH-NAD and then inhibits mycolic acid biosynthesis by binding the active site of the FAS-II enoyl-ACP reductase InhA.

### 1.2.5 Known ways to enhance INH activity

Since INH is a cornerstone drug in frontline tuberculosis therapy, identifying ways to enhance its antimycobacterial activity could dramatically improve the current antimicrobial regimen. It has been shown that in a non-growing state, such as during stationary phase or incubation in hypoxic conditions, \textit{Mtb} becomes tolerant to the bactericidal effects of INH\textsuperscript{30,118}. Inhibiting the hypoxia-responsive two-component signaling system DosRST can prevent \textit{Mtb} from adopting a drug tolerant state, thereby sensitizing \textit{Mtb} in these conditions to killing by INH\textsuperscript{119}. Furthermore, using the mycobacterial respiration inhibitors bedaquiline (BDQ), which targets ATP synthase, Q203, which targets the cytochrome bc\textsubscript{1}, and CCCP, which is an ionophore that disrupts the proton motive force, it was demonstrated that inhibition of the ETC antagonizes the bactericidal effect of INH\textsuperscript{120–122}. These findings suggest that decreases in \textit{Mtb} respiration, either when the bacteria encounter a hypoxic environment or are exposed to ETC inhibitors, prevent INH from exerting its full bactericidal effect. Consistent with this model, it was shown that providing \textit{Mtb} with exogenous reducing agents can enhance the bactericidal activity of INH, likely by increasing \textit{Mtb} respiration through the donation of electrons from the reducing agent directly into the ETC and preventing the bacteria from adopting a drug tolerant dormant state\textsuperscript{19}. The underlying mechanism that connects ETC activity to INH killing is not well understood, but could be a result of the decreased cell expansion rate that occurs in energy-limiting conditions. It is known from studies in model organisms that cell wall inhibitors exhibit the most effective bactericidal activity.
when cells are actively expanding and growing. This model is supported by single-cell studies demonstrating that faster elongating mycobacterial cells within a population are more susceptible to INH compared to slower elongating cells.

However, not all respiration inhibitors antagonize killing by INH. For example, an inhibitor of the menaquinone biosynthesis enzyme MenG causes a block in \textit{Mtb} oxygen consumption, similar to other respiration inhibitors, yet actually enhances the bactericidal effect of INH. This example of a small molecule that can potentiate killing by INH in spite of inhibiting \textit{Mtb} energy metabolism highlights that there remain gaps in our understanding of how INH sensitivity is connected to \textit{Mtb} energy metabolism.

Multiple groups have used genetic screening approaches to discover mechanisms to enhance the activity of INH. In one approach, a group generated set of 207 \textit{Mtb} strains, each harboring a plasmid that encodes a predicted transcriptional regulator under an inducible promoter, and then screened these strains to identify transcription factors that can promote sensitivity to INH. Through these efforts, it was discovered that inducing the expression of the gene \textit{mce3R} causes enhanced sensitivity to INH through repression of the gene \textit{ctpD}. Transposon insertion within \textit{ctpD} leads to enhanced killing by INH, independent of whether \textit{mce3R} is induced, suggesting that the effects of \textit{ctpD} are downstream of \textit{mce3R}. Furthermore, repressing \textit{ctpD} expression through over-expression of \textit{mce3R} or disruption of \textit{ctpD} caused elevated accumulation of activated INH-NAD in INH-treated bacteria. This finding suggests that disruption of \textit{ctpD} enhances the bacterial sensitivity to INH by increasing the level of active INH-NAD within the cell. CtpD is a metal ion transporter, that can export Fe\textsuperscript{2+} from the cell, leading the authors to speculate that repression or disruption of \textit{ctpD} causes intracellular Fe\textsuperscript{2+} accumulation, and perhaps increased iron-dependent KatG expression or activity.
Another group used a transposon insertion-sequencing (Tn-Seq) approach to identify mutants that have enhanced INH sensitivity and found that disruption of the gene cinA causes enhanced killing by INH \(^{126}\). A \(\Delta\)cinA mutant accumulated elevated INH-NAD levels \(^{126}\). CinA harbors a deamidase domain and a pyrophosphatase domain, and the authors demonstrate that CinA acts as an INH-NAD degrading enzyme, cleaving the INH-NAD adduct. Therefore, the \(\Delta\)cinA mutant is more sensitive to INH because it is unable to degrade INH-NAD \(^{126}\).

A third screening-based approach used a pooled CRISPR-interference (CRISPRi) library to conditionally repress 4,052 of the 4,125 genes in \(Mtb\), culturing the library in the presence of antibiotics such as INH to identify knock-down strains that are enriched or depleted from the population \(^{127}\). The authors identified 196 genes that led to enrichment in the presence of INH when targeted by CRISPRi, suggesting that knocking down these genes leads to INH-resistance. This gene set included \(katG\), and other genes that are known to mediate INH-resistance \(^{127}\). Additionally, the authors identified 230 genes that when knocked down by CRISPRi caused hypersensitivity to INH. These genes included cinA, corroborating the Tn-seq study that originally identified the role of this gene in INH sensitivity \(^{126}\). Additionally, the authors found that depletion of \(inhA\) sensitized \(Mtb\) to INH, as expected since depletion of its target should titrate the amount of INH required to inhibit the bacteria \(^{127}\). In addition to corroborating known chemical-genetic interactions, these systems-level approaches have the capacity to identify novel factors that modulate INH sensitivity \(^{127–129}\).

### 1.3 Addressing \(Mtb\) drug resistance

#### 1.3.1 Small molecules that reverse resistance

Resistance to antibiotics is a dire threat. In addition to enhancing the activity of antibiotics
against WT Mtb to shorten our antibiotic regimens and curtail the emergence of resistant isolates during infection, it is critical to identify ways to target these resistant strains. One approach to circumvent Mtb drug resistance is to develop small molecules that re-sensitize resistant strains to antibiotics. This approach has been successfully implemented in the treatment of several pathogenic bacteria. However, there are very few examples of small molecules that reverse resistance in Mtb, and none of these have yet been successfully introduced into the clinic, suggesting that there is work to be done to improve this approach for the treatment of tuberculosis.

The vast majority of small molecules that reverse bacterial drug resistance have been shown to function by increasing the concentration of active antibiotic within the bacterial cell. Perhaps the most successful implementation of this strategy is the use of β-lactamase inhibitors in combination with β-lactam antibiotics. While β-lactams are commonly used to treat a variety of bacterial infections, this class of antibiotics is typically not used in the treatment of Mtb largely because Mtb harbors an endogenous β-lactamase gene that renders the bacterium naturally resistant to β-lactams. The β-lactamase inhibitor clavulanate was discovered to enhance the activity of β-lactams against bacterial strains that have acquired a β-lactamase resistance gene. Since the discovery of clavulanate, a number of additional small molecules have been discovered that inhibit or inactivate β-lactamases through various mechanisms, highlighting the importance of this classic resistance reversal strategy. Treating Mtb with clavulanate in combination with the β-lactam meropenem can overcome its natural resistance and result in killing of Mtb both in vitro and in mice. However, the combination of meropenem and clavulanate has not been adopted in the clinic to treat Mtb infections because the toxicity of long-term meropenem use renders this therapy untenable.

Another way to re-sensitize resistant strains is to increase the influx of the antibiotic or
decrease efflux allowing the antibiotic to reach the critical concentration that overcomes the resistance mechanism in the strain. For example, compounds that permeabilize the outer membrane of Gram-negative bacteria can increase the concentration of drug at the site of action and enhance the sensitivity of the bacteria to an antibiotic even in resistant isolates \(^\text{140,141}\). In a similar approach, compounds that block bacterial efflux pumps can increase the intracellular concentration of antibiotic and overcome resistance in some cases \(^\text{142}\), which is an approach that has some success in enhancing antibiotic efficacy against \(Mtb\) \(^\text{143}\).

In addition to inhibiting drug degradation or promoting drug accumulation, a third strategy to increase the concentration of active antibiotic involves enhancing the activation of a pro-drug. The anti-\(Mtb\) antibiotic ethionamide (ETH) is primarily activated by the enzymes EthA and MymA \(^\text{144,145}\). Genetic disruption of either \(ethA\) or \(mymA\) confers a 5.7- or 4.6-fold increase in the IC\(_{50}\) of ETH respectively, but deletion of both enzymes confers a >20-fold increase in the IC\(_{50}\) \(^\text{145}\). Mutations in \(ethA\) or in a gene that encodes its transcriptional activator, \(ethR\), are commonly associated with clinical ETH resistance \(^\text{146}\). It was recently discovered that small molecules that target transcriptional regulators to induce the expression of \(mymA\) or induce the expression of a cryptic activation enzyme encoded by \(ethA2\) in \(ethA\) mutant bacteria can re-sensitize these resistant strains to ETH \(^\text{147,148}\). These studies indicate that modulating the concentration of active antibiotic is a promising approach to overcoming antibiotic resistance.

Ultimately, there are very few examples of small molecules that reverse \(Mtb\) drug resistance, and these approaches rely on the strategy of increasing the active concentration of antibiotic, either by blocking the activity of endogenous \(\beta\)-lactamases to prevent degradation of \(\beta\)-lactam drugs, which are not currently in use in the clinic \(^\text{130}\), or by inducing expression of ETH-activating enzymes to increase the activation of the second line antibiotic ETH \(^\text{147,148}\). Recently,
our lab reported that the compound C10 has the remarkable capacity to re-sensitize INH-resistant \textit{katG} mutants to inhibition by this frontline antibiotic \cite{45}. While the mechanism of action of C10 remains unknown, we present evidence in Chapter 3 that C10 re-sensitizes INH-resistant \textit{Mtb} without changing the concentration of active INH-NAD, representing a new paradigm in strategies to reverse \textit{Mtb} drug resistance.

\textbf{1.3.2 Inhibitors with novel mechanisms of action – the ETC as a drug target}

In addition to identifying small molecules that reverse resistance, the discovery of antibiotics with novel mechanisms of action is a promising approach to target resistant isolates. In 2012, the diarylquinoline compound bedaquiline (BDQ), under the name of Sirturo, was approved to treat multidrug-resistant \textit{Mtb} infections, which represented the first new tuberculosis antibiotic approved in decades \cite{149}. BDQ inhibits the mycobacterial ATP synthase, the terminal enzyme in the oxidative phosphorylation pathway \cite{150}. Unfortunately, immediately after its implementation in the clinic, it became apparent that BDQ causes life-threatening liver toxicity, so its use is reserved only for last-resort treatment \cite{151}. Still, the success of this new antibiotic fueled intense interest in mycobacterial energy metabolism as a promising target for antibiotic development.

\textbf{1.3.3 The mycobacterial oxidative phosphorylation pathway}

\textit{Mtb} is an obligate aerobic bacterium, and while it can survive prolonged periods in hypoxic conditions, it cannot grow unless it has access to oxygen \cite{30}. Oxidative phosphorylation through the electron transport chain and ATP synthase is essential for \textit{Mtb} survival, as mycobacteria cannot generate sufficient ATP by substrate level phosphorylation to meet energy demands \cite{152}. ATP synthase activity is dependent on the proton motive force, which is generated and maintained by the ETC. Two classes of enzymes are required for ETC activity: dehydrogenases and terminal oxidases. Respiratory dehydrogenases transfer electrons from reduced cofactors (e.g. NADH or
FADH$_2$) to menaquinone, a lipid electron carrier, reducing menaquinone to menaquinol. The terminal oxidases then transfer electrons from menaquinol to oxygen, the terminal electron acceptor. Each of these electron transfer reactions has the capacity to drive translocation of protons across the membrane, establishing the proton gradient.

There are nearly a dozen dehydrogenases that are known or predicted to supply electrons to the ETC in Mtb $^{153}$. However, Mtb only has two terminal oxidases, the cytochrome $bc_1$:$aa_3$ oxidase encoded by $qcrCAB$ and $ctaBCDE$ and the cytochrome $bd$ oxidase encoded by $cydAB$ $^{154,155}$. This branched ETC allows Mtb to shuttle electrons either to cytochrome $bc_1$:$aa_3$ or to cytochrome $bd$ to be transferred to the terminal electron acceptor, O$_2$ $^{155}$. Cytochrome $bc_1$:$aa_3$ is proposed to be bioenergetically more efficient than cytochrome $bd$ in aerobic conditions. As such, cytochrome $bc_1$:$aa_3$ is important for growth of Mtb under standard laboratory conditions.

The imidazopyridine amide Q203 (also known as Telacebec) was identified to be a potent antimycobacterial compound that targets QcrB, a subunit of the mycobacterial cytochrome $bc_1$:$aa_3$ oxidoreductase in the ETC $^{156}$. A variety of compounds have subsequently been discovered that target this complex, suggesting that this enzyme may be a promiscuous target $^{157–163}$. Clinical trial data shows that Q203 has promising efficacy in human patients, although in monotherapy it is not nearly as effective as the standard treatment regimen $^{164}$. Data from a mouse model of infection suggests that cytochrome $bc_1$:$aa_3$ inhibitors can halt Mtb growth in the mouse when administered during the acute phase of infection, yet have a variable impact on bacterial burden when treatment is started after Mtb has already established an infection, with most studies reporting no decrease or modest decreases in the bacterial burden at this later stage $^{156,159,160,165–167}$. This could be because cytochrome $bc_1$:$aa_3$ expression is down-regulated and cytochrome $bd$ is up-regulated significantly after the first 20 days of infection $^{168}$. These findings suggest that Mtb may rely more heavily on
cytochrome $bc_1:aa_3$ during early stages of infection, whereas cytochrome $bd$ is activated at later stages of infection.

Deletion of cytochrome $bd$ dramatically sensitizes $Mtb$ to killing by cytochrome $bc_1:aa_3$ inhibitors, indicating that it is possible to overcome this ETC plasticity $^{157,166}$. Furthermore, exposing $Mtb$ simultaneously to cytochrome $bc_1:aa_3$ inhibitors in combination with specific inhibitors of cytochrome $bd$ leads to enhanced bacterial killing $^{169-172}$, suggesting that combination therapy with cytochrome $bd$ inhibitors could promote the efficacy of cytochrome $bc_1:aa_3$ inhibitors in the clinic.

1.4 Aim and Scope of the Dissertation

Given the immense public health burden incurred by $Mtb$ drug tolerance and drug resistance, this dissertation describes a series of approaches that led to the identification of a small molecule that enhances $Mtb$ antibiotic sensitivity and reverses drug resistance (Chapters 2-4) as well the discovery of a novel chemical class of ETC inhibitors (Chapter 5).

In Chapter 2, I describe a novel chemical screening approach that led to the discovery of the compound C10, which we demonstrated can block tolerance to the important antibiotic INH and can even re-sensitize INH-resistant $katG$ mutants in vitro. Through transcriptional profiling, we determined that C10 perturbs $Mtb$ energy metabolism, although it remained unclear if this perturbation caused the enhanced INH sensitivity. Additionally, the precise mechanism of action of C10 was unknown. In Chapter 3, we address this question by isolating C10-resistant mutants and recovering isolates that were resistant to the effects of C10 on energy homeostasis. Using these mutants in combination with chemical tools, we reveal that the inhibition of energy metabolism by C10 is not required or sufficient for C10 to enhance INH sensitivity. Using a combination of
forward genetics and chemical biology, we provide evidence that C10 restores INH sensitivity in a subset of resistant mutants through a previously un-described mechanism. While the target and exact mechanism of action of C10 remains enigmatic, these studies provide important insight into the pleiotropic effects of C10 on \textit{Mtb} physiology. Since energy metabolism pathways represent promising drug targets, Chapter 4 describes our attempts to characterize how C10 inhibits energy metabolism in \textit{Mtb}.

Finally, Chapter 5 describes a strategy to screen for novel anti-\textit{Mtb} inhibitors, in which we identify 4-amino-thienopyrimidines as a novel chemical class of \textit{Mtb} inhibitors. Through forward genetics and assays to measure bacterial bioenergetics, we provide evidence that these compounds target \textit{Mtb} oxidative phosphorylation by inhibiting the QcrB subunit of cytochrome \textit{bc1:aa3}, revealing a new chemical scaffold that targets this validated drug target. Together, these chapters summarize a body of work that reveals bacterial vulnerabilities that can be exploited to inhibit resistant \textit{Mtb} and highlights the importance of energy metabolism as a target for \textit{Mtb} drug development.
Chapter 2: Chemical disarming of isoniazid resistance in *Mycobacterium tuberculosis*

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Preface:

A version of this chapter was previously published in *PNAS* in 2019 as a co-first author study between GAH and KF. To clarify our respective contributions in this chapter, each figure legend will list the initials of the author that generated the data and attribute each panel in the figure to the appropriate contributor. The majority of the data presented in this chapter was generated by GAH or KF under the supervision of CLS, with a few exceptions. JL and DXZ contributed to the RNA-sequencing analysis. The compound library that was screened and the lead compound C10 that is used in this study were designed and synthesized by a team of chemists: JADG, MRH, AGC, MK, TW, AEGL, EC, CB, KSK, SS, and FA Additionally, MES and RLK assisted with cloning the ΔcydA deletion construct.
2.1 Abstract:

Before the COVID-19 pandemic, *Mycobacterium tuberculosis* (*Mtb*) killed more people each year than any other single infectious agent. This dangerous pathogen is able to withstand stresses imposed by the immune system and tolerate exposure to antibiotics, resulting in a persistent infection. The global tuberculosis (TB) epidemic has been exacerbated by the emergence of mutant strains of *Mtb* that are resistant to frontline antibiotics. Thus, both phenotypic drug tolerance and genetic drug resistance are major obstacles to successful TB therapy. Using a chemical approach to identify compounds that block stress and drug tolerance, as opposed to traditional screens for compounds that kill *Mtb*, we identified a small molecule (C10) that blocks tolerance to oxidative stress, acid stress, and the frontline antibiotic isoniazid (INH). In addition, we found that C10 prevents the selection for INH-resistant mutants and restores INH sensitivity in otherwise INH-resistant *Mtb* strains harboring mutations in the *katG* gene, which encodes the enzyme that converts the pro-drug INH to its active form. Through mechanistic studies we discovered that C10 inhibits *Mtb* respiration, revealing a link between respiration homeostasis and INH sensitivity. Therefore, by using C10 to dissect *Mtb* persistence, we discovered that INH resistance is not absolute and can be reversed.

2.2 Significance Statement:

*Mycobacterium tuberculosis* (*Mtb*) causes the disease tuberculosis (TB), which before COVID-19 killed more people than any other infection. The emergence of drug-resistant *Mtb* strains has exacerbated this already alarming epidemic. We have identified a small molecule, C10, that potentiates the activity of the frontline antibiotic isoniazid (INH) and prevents the selection for INH-resistant mutants. We find that C10 can even reverse INH resistance in *Mtb*, which has
never before been reported. Therefore, our study has revealed that there are vulnerabilities that can be exploited to reverse INH resistance in \textit{Mtb}.

2.3 Introduction:

As one of the deadliest pathogens in the world, \textit{Mycobacterium tuberculosis} (\textit{Mtb}) infections cause 1-2 million deaths each year \cite{44}. During infection, \textit{Mtb} is exposed to an arsenal of host-derived stresses. However, the bacteria respond to stress with physiological changes that allow \textit{Mtb} to tolerate these immune stresses and persist \cite{175}. These same physiological changes result in antibiotic tolerance where the bacteria are genetically susceptible to antibiotics, but exist in a physiological state rendering them recalcitrant to therapy \cite{17,38,174,175}. As a result, long courses of antibiotic therapy are required to treat tuberculosis (TB) \cite{3}, leading to the emergence of drug-resistant mutant strains of \textit{Mtb}. In 2017, out of the 10 million cases of TB, an estimated 19\% of newly treated cases and 43\% of previously treated cases exhibited resistance to at least one of the frontline antibiotics \cite{44}. Resistance to the frontline antibiotic isoniazid (INH) is the most common form of \textit{Mtb} mono-resistance, and is associated with treatment failure, relapse, and progression to multidrug-resistant TB \cite{44}. Together, the problems of phenotypic tolerance and genetic resistance to antibiotics undermine current TB treatment options. There is a dire need for new strategies that shorten the duration of treatment and target both drug-tolerant and genetically drug-resistant \textit{Mtb}, which requires a better understanding of how \textit{Mtb} survives exposure to immune defenses and antibiotic therapy.

Previous work has demonstrated that a number of stresses are capable of inducing the formation of drug tolerant \textit{Mtb} \cite{30,34,176}. The most thoroughly studied inducer of drug tolerance is hypoxia. Exposure to hypoxic conditions has pleiotropic effects on the bacteria, including
replication arrest, induced expression of dormancy-associated genes, shifts in *Mtb* lipid composition, and global shifts in *Mtb* metabolism and respiration. However, it remains unclear mechanistically how these changes in physiology confer tolerance to stress and antibiotics.

To address this gap in understanding, we developed a chemical screen to identify compounds that inhibit the development of hypoxia-induced stress and drug tolerance. Through this chemical approach, we identified a compound, C10, that inhibits the development of hypoxia-induced tolerance to oxidative stress and INH. In addition to blocking tolerance, we found that C10 prevented the selection for INH-resistant mutants and re-sensitized an INH-resistant mutant to INH, providing the first evidence that INH resistance can be reversed in *Mtb*.

### 2.4 Results:

#### 2.4.1 C10 blocks hypoxia-induced tolerance to oxidative stress and INH

To dissect mechanisms of persistence, we used a modified version of the culture-based hypoxia model that is routinely used to study *Mtb* drug tolerance. We incubated *Mtb* in liquid media for 3 weeks in airtight containers. During this incubation, oxygen levels drop and drug-tolerant bacteria develop. We then re-aerated the cultures for 2 more weeks, during which time *Mtb* forms a pellicle biofilm at the air-liquid interface. Using this model, we performed a screen for chemical inhibitors of pellicle formation. We chose a library of 91 compounds that shared a peptidomimetic bicyclic central fragment (a thiazolo ring-fused 2-pyridone, Figure 2.1A). Previous work has shown that depending on the substituents introduced to the 2-pyridone scaffold, compounds within this library exhibit diverse but highly specific biological activities, including some compounds that inhibit pellicle formation in *Escherichia coli*. From this screen, we identified 12 compounds that inhibited *Mtb* pellicle formation at 10μM, the most potent
of which was C10 (Figure 2.1B)\textsuperscript{188}. C10 inhibited \textit{Mtb} pellicle formation (Figure 2.1C) with a minimum inhibitory concentration (MIC) of 6.25\textmu M (Supplementary Figure 2.S1). Despite the absence of a pellicle, wells treated with C10 contained >1x10\textsuperscript{7} colony forming units (CFU) per mL (Figure 2.1D). Therefore, the absence of a pellicle was not due to a lack of viable bacteria; C10 specifically inhibits a physiological process required for pellicle formation.

Since hypoxia promotes drug tolerance as well as pellicle formation\textsuperscript{30,181}, we reasoned that small molecule pellicle inhibitors may target physiological processes linked to stress and drug tolerance. Therefore, we first examined the effect of C10 on sensitivity to reactive oxygen species (ROS), since mycobacteria upregulate transcripts to cope with ROS in pellicles\textsuperscript{189}. We cultured \textit{Mtb} in hypoxic conditions for 3 weeks ±C10, then re-aerated the cultures and added hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) to induce oxidative stress for 2 weeks (Figure 2.1E-F). In the absence of C10, \textit{Mtb} survived exposure to up to 100 mM H\textsubscript{2}O\textsubscript{2} (Figure 2.1F, DMSO). In contrast, exposure of C10-treated cultures to 100 mM H\textsubscript{2}O\textsubscript{2} resulted in a reduction in CFU below the limit of detection, demonstrating that C10 blocks hypoxia-induced tolerance to H\textsubscript{2}O\textsubscript{2} (Figure 2.1F).

We then tested whether C10 affected hypoxia-induced antibiotic tolerance, starting with the frontline antibiotic INH, which targets mycolic acid biosynthesis\textsuperscript{54}. During infection, \textit{Mtb} becomes phenotypically tolerant to INH, which can be reproduced \textit{in vitro} by culturing \textit{Mtb} in low oxygen\textsuperscript{17,30,174}. We incubated \textit{Mtb} in hypoxic conditions for 3 weeks ±C10 before re-aerating the cultures and adding INH for 2 additional weeks (Figure 2.1E and G). The survival of DMSO-treated control cultures decreased with increasing INH concentrations (Figure 2.1G, DMSO). However, even at the highest INH concentration (1000\textmu g/mL), a population of \textit{Mtb} remained viable, similar to previous reports\textsuperscript{181}. The presence of C10 led to a dramatic decrease in survival following INH treatment (Figure 2.1G). Exposure to 200\textmu g/mL INH only killed ~1.5 logs of
DMSO-treated *Mtb*, whereas there were no culturable bacteria detected in C10-treated samples exposed to 200μg/mL INH. Thus, C10 blocks the ability of *Mtb* to develop hypoxia-induced INH tolerance. In contrast, C10 did not significantly affect *Mtb* sensitivity to rifampicin, streptomycin, or ethambutol, which inhibit RNA polymerase, the ribosome, and arabinogalactan synthesis, respectively 190–192 (Supplementary Figure 2.S2).

2.4.2 C10 potentiates killing by INH and prevents the selection for INH resistant mutants

The striking and specific effects of C10 on INH tolerance indicated that C10 uniquely potentiates INH. To test if C10 had a general effect on INH sensitivity, or if it specifically blocks hypoxia-induced INH tolerance, we cultured *Mtb* in planktonic, aerated conditions in media containing C10 and/or INH, and monitored growth by changes in optical density (OD$_{600}$)(Figure 2.2A). In these conditions, treatment with 5μM C10 alone resulted in no difference in growth compared to the DMSO-treated control and treatment with 25μM C10 resulted in a 1.53-fold increase in *Mtb* doubling time (Figure 2.2A and B). Since we used an INH concentration above the MIC (0.02-0.04μg/mL 193), INH treatment inhibited *Mtb* growth with or without C10. We enumerated the surviving CFU after 10 days of treatment by plating the viable bacteria on agar media without drugs and found that the addition of C10 in combination with INH resulted in a significant and dose-dependent decrease in viable bacteria compared to INH alone. Therefore, C10 potentiates the bactericidal activity of INH against aerobically grown *Mtb* over a 10 day treatment period (Figure 2.2C).

To further study the impact of C10 on INH efficacy, we spread ~8X10$^7$ CFU of *Mtb* on agar media containing C10 and/or INH so that the bacteria are continually exposed to the drugs as opposed to the transient 10 day exposure in liquid culture. *Mtb* formed a lawn of bacterial growth
on agar containing DMSO or 25μM C10 (Figure 2.2D). Growth of Mtb on agar containing 0.5μg/mL INH was inhibited, with the exception of spontaneous INH-resistant colonies that emerged at an approximate frequency of 1 in 10^6, similar to previous reports. In contrast, when C10 was present in combination with INH, no resistant colonies grew, demonstrating that C10 blocked the selection for INH-resistant mutants (Figure 2.2D).

### 2.4.3 C10 re-sensitizes katG mutant Mtb to inhibition by INH

The majority of INH-resistant clinical isolates harbor mutations in katG, which encodes the sole catalase-peroxidase in Mtb and the enzyme that converts INH into its active form. We sequenced the katG gene from seven of the colonies that grew on agar containing INH (Figure 2.2D) and identified katG mutations in all seven isolates: four harbored frameshift mutations and three had missense mutations (Supplementary Table 2.S1). Since no INH-resistant katG mutants grew when C10 was combined with INH (Figure 2.2D), the growth of the katG mutants must be inhibited by either C10 alone or the C10-INH combination. To distinguish between these possibilities, we monitored the growth of an Mtb isolate with a frameshift mutation at amino acid 6 in katG (katG^FS) in aerated, planktonic cultures in the presence of C10 and/or INH (Figure 2.3A). As expected, the INH-resistant katG^FS mutant was able to grow in media containing INH (doubling time 3.28 ± 0.20 days), albeit 1.23-fold slower than the DMSO treated cultures (doubling time 2.66 ± 0.20 days)(Figure 2.3B). 5μM C10 did not significantly affect the growth rate of the katG^FS strain and 25μM C10 increased the doubling time of the katG^FS strain 1.47-fold (Figure 2.3B), which is comparable to the 1.53-fold increase in doubling time caused by 25μM C10 in WT Mtb (Figure 2.2B). Therefore, the katG^FS strain is not significantly more sensitive than WT Mtb to treatment with C10 alone. However, the combination of C10 and INH significantly inhibited growth of the katG^FS strain as compared to INH or C10 alone (Figure 2.3A). We enumerated viable
bacteria from these cultures after 10 days of treatment by plating the surviving bacteria on agar media without drugs and found that although INH or C10 alone did not significantly decrease the number of surviving katG\textsuperscript{FS} \textit{Mtb}, the combination of C10 and INH resulted in a significant reduction in CFU/mL (Figure 2.3C), further demonstrating that the C10-INH combination inhibits the katG\textsuperscript{FS} mutant. Similarly, the katG\textsuperscript{FS} mutant grew on agar containing either C10 or INH alone, but not on agar containing the combination of both C10 and INH (Figure 2.3D). Therefore, C10 restored the sensitivity of the katG\textsuperscript{FS} mutant to INH.

The absence of growth of any katG mutants on plates containing INH and C10 (Figure 2.2D) suggests that C10 restores the sensitivity of all katG mutants that would normally be selected for in the presence of INH alone. To directly test if the effect of C10 can be generalized to additional INH-resistant katG mutants, we measured the impact of C10 on INH sensitivity in two additional strains harboring mutations in katG at residues that have been identified as mutated in INH-resistant clinical isolates: katG\textsuperscript{A172T} and katG\textsuperscript{W328L} \textsuperscript{195,196}. When we treated the katG\textsuperscript{A172T} and katG\textsuperscript{W328L} \textit{Mtb} mutants with 5μM C10 and/or 0.25μg/mL INH, we found that the combination of both C10 and INH resulted in significantly decreased growth compared to either treatment alone, similar to the katG\textsuperscript{FS} mutant (Figure 2.3E-F). These studies provide evidence that INH resistance can be reversed in katG mutant strains of \textit{Mtb}.

2.4.4 C10 perturbs \textit{Mtb} metabolism and respiration

To decipher the mechanism by which C10 sensitizes \textit{Mtb} to INH, we compared gene expression profiles of WT \textit{Mtb} treated with 5μM or 25μM C10 for 48 hours in aerated conditions to DMSO-treated control \textit{Mtb} using RNA-sequencing (RNA-seq). Treatment with 5μM C10 resulted in significant (p\textsubscript{adj} < 0.05) up-regulation of only 12 genes greater than 1.5-fold, whereas treatment with 25μM C10 caused significant up-regulation of 194 genes greater than 1.5-fold,
including 9 of the genes upregulated in 5μM C10. When we classified the genes induced by C10 into functional categories based on their annotation in Mycobrowser, we found that the functional category with the most genes upregulated by 25μM C10 treatment in aerobic conditions was intermediary metabolism and respiration (Figure 2.4A). In addition, the gene Rv0560c, which encodes a putative benzoquinone methyltransferase that may be involved in synthesis or modification of the electron transport chain carrier benzoquinone, was one of the two most highly up-regulated genes in both 5μM and 25μM C10 treatments. We also found that all of the genes encoding the cytochrome bd complex (cydABDC) were significantly upregulated greater than 1.5-fold in Mtb treated with 25μM C10, which is a hallmark of respiration inhibition. Multiple other electron transport (ETC) genes were also significantly induced by 25μM C10 but did not meet the 1.5-fold cut off.

We performed a similar gene expression analysis of Mtb cultured for two weeks in low oxygen ± 50μM C10 and found that C10 caused significant up-regulation of 716 genes greater than 1.5-fold (p_{adj} < 0.05). Our finding that C10 caused up-regulation of more genes in hypoxic conditions compared to aerobic conditions may reflect the different concentrations and timing of C10 treatment in these two conditions. Alternatively, Mtb may be more sensitive to C10 treatment in hypoxic conditions, although 50μM C10 has no effect on Mtb survival in hypoxia (Figure 2.1D). Similar to in aerated cultures treated with 25μM C10, the functional category with the most genes up-regulated was intermediary metabolism and respiration (148 genes) (Supplementary Figure 2.S3). In addition, like we had observed in aerobic cultures treated with 25μM C10, we found that Rv0560c was the most highly up-regulated gene and multiple other ETC genes were up-regulated greater than 1.5-fold, including cydABDC. These data indicate that C10 affects similar pathways in both hypoxic and aerated conditions. Notably, we found that C10 did not inhibit induction of
the DosR “dormancy regulon” that is up-regulated in hypoxia \(^{177}\), demonstrating that C10 does not inhibit the ability of \(Mtb\) to sense or respond to hypoxia (Supplementary Table 2.S2 and Supplementary Figure 2.S4).

Since treatment with C10 in both normoxic and hypoxic conditions led to upregulation of genes encoding components of the ETC, we examined whether C10 affects respiration by monitoring oxygen consumption by \(Mtb\) using methylene blue dye. In the presence of oxygen, this dye is blue, but when incubated with \(Mtb\) for 16 hours in an airtight tube, oxygen is consumed and the dye becomes reduced and turns colorless \(^{198}\). Addition of 2.5\(\mu\)g/mL INH, an antibiotic that does not target respiration, did not affect methylene blue decolorization by \(Mtb\). In contrast, treatment with 50\(\mu\)M C10 blocked methylene blue decolorization, similar to clofazimine (CFZ), which inhibits type II NADH dehydrogenases in the ETC \(^{199}\) (Figure 2.4B). Bacterial viability was similar in all conditions (Figure 2.4C), demonstrating that inhibition of methylene blue decolorization was not a secondary effect of killing the bacteria and that C10 blocked oxygen consumption. To quantify the minimum concentration of C10 required to perturb metabolism or respiration, we examined the activity of C10 in the Microplate Alamar Blue Assay (MABA) \(^{200}\). The MABA utilizes the dye resazurin, which is blue in its oxidized form but is reduced to the pink fluorescent compound resorufin as a result of cellular metabolism. The MABA is commonly used to evaluate the efficacy of anti-mycobacterial compounds, but also serves as a measure of metabolism and respiration. C10 inhibited the reduction of resazurin with a half-maximal inhibitory concentration (IC\(_{50}\)) of 8.2 ± 1.3\(\mu\)M (Figure 2.4D).

A limitation of the methylene blue and resazurin-based assays is that they both rely on redox-sensitive dyes as an indirect readout of metabolism and/or respiration. To confirm that C10 affects energy metabolism, we examined the effect of C10 treatment on ATP levels in the bacteria
by treating *Mtb* with 5μM or 25μM C10 for 24 hours and then measuring ATP levels using the luciferase-based BacTiter Glo assay. Indeed, C10 treatment caused a significant dose-responsive decrease in ATP as compared to the DMSO-treated cultures (Figure 2.4E). Notably, treatment with C10 in combination with INH gave very similar results to C10 alone, demonstrating that INH does not enhance the ability of C10 to deplete *Mtb* ATP levels (Figure 2.4E). As expected by the dose-responsive effects of C10 on ATP levels, increasing the concentration of C10 to 100μM or 250μM resulted in complete inhibition of growth (Supplementary Figure 2.S5), likely due at least in part to depletion of ATP.

Perturbations in respiration can result in the production of ROS \(^{19,199}\), and ROS have been shown to sensitize bacteria to INH \(^{201-203}\). Therefore, if C10 treatment leads to the generation of ROS, this could contribute to increased sensitivity of *Mtb* to INH. To test the effect of C10 treatment on the production of ROS, we used the ROS-sensitive dye CellROX Green, which becomes oxidized upon exposure to ROS, resulting in a fluorescent product. We treated *Mtb* with 5μM or 25μM C10 for 24 hours before staining with CellROX Green and measuring fluorescence as a read-out for ROS. Treatment of *Mtb* with 1μg/mL CFZ, a known inducer of ROS \(^{204}\), increased CellROX fluorescence. In contrast, treatment with C10 did not increase CellROX fluorescence (Figure 2.4F), demonstrating that C10 does not cause accumulation of ROS in WT *Mtb*. Addition of INH to the C10-treated cultures also did not result in a change in CellROX fluorescence, indicating that the effects of C10 on INH sensitivity in WT *Mtb* are not mediated through ROS accumulation.

### 2.4.5 C10 sensitizes *Mtb* to acid stress

Since respiration plays an important role in maintaining intrabacterial pH homeostasis \(^{205-207}\), we hypothesized that inhibition of respiration by C10 could compromise the ability of *Mtb* to
survive exposure to acid stress. We tested if C10 sensitizes \textit{Mtb} to low pH by culturing \textit{Mtb} aerobically in media at pH 7.0 or 5.5 and monitoring bacterial survival. In the absence of C10, \textit{Mtb} cultured at pH 5.5 for 8 days showed no loss of viability. In contrast, in the presence of C10, the viability of \textit{Mtb} cultured at pH 5.5 decreased greater than 3 orders of magnitude over 8 days (Figure 2.5A), demonstrating that C10 sensitizes \textit{Mtb} to low pH. In addition, C10 inhibited growth of \textit{Mtb} on low pH agar media (Figure 2.5B), further demonstrating that C10 sensitizes \textit{Mtb} to acid stress, consistent with our findings that C10 perturbs respiration.

\textbf{2.4.6 C10 potentiates killing by the clinical candidate Q203 without targeting the cytochrome complexes}

A hallmark of respiration inhibitors is their ability to synergize with genetic or chemical inhibition of parallel complexes in the ETC\textsuperscript{166,204}. Therefore, we inquired whether C10 would enhance the activity of the ETC inhibitor Q203, which targets cytochrome $bc_1$ and is currently in clinical trials for TB treatment\textsuperscript{156}. We incubated \textit{Mtb} with 25\textmu M C10, 400 nM Q203, or the combination in liquid cultures and enumerated viable bacteria after 15 days of treatment by plating the surviving bacteria on agar media without drugs (Figure 2.6A-B). We did not observe any reduction in viable bacteria in cultures treated with C10 or Q203 alone. However, the combination of C10 and Q203 resulted in a significant decrease in \textit{Mtb} viability after 15 days of treatment. These findings show that the combination of C10 with Q203 results in bactericidal activity within 15 days of treatment and supports our hypothesis that C10 inhibits respiration. \textit{Mtb} encodes two cytochromes, cytochrome $bc_1$ and cytochrome $bd$, that display some redundancy in function. As such, genetic inactivation of cytochrome $bd$ results in hypersensitivity to inhibition of cytochrome $bc_1$ with Q203\textsuperscript{166,204}. Since C10 induced expression of $\text{cydABDC}$ and also potentiated killing by Q203, we inquired whether C10 directly targeted cytochrome $bd$ by treating a $\Delta \text{cydA}$ \textit{Mtb} strain...
with C10 and monitoring effects on metabolism and respiration in the MABA. We found that C10 inhibited ΔcydA \textit{Mtb} to a similar extent as WT \textit{Mtb} (Supplementary Figure 2.S6), demonstrating that C10 does not directly target cytochrome \textit{bd} and potentiates Q203 activity via a different mechanism. These data also demonstrate that C10 does not directly target cytochrome \textit{bc}_1 because inhibitors of this cytochrome have increased activity in \textit{cydAB} mutants\textsuperscript{166,204}.

2.5 Discussion:

The studies presented herein uniquely exploit the link between the development of drug tolerance and pellicle biofilm formation to identify compounds that inhibit pathways that contribute to drug and stress tolerance. Instead of screening for compounds that kill \textit{Mtb}, we identified inhibitors of biofilm formation and found C10, a compound that sensitizes \textit{Mtb} to the antibiotics INH and Q203 as well as the physiologically relevant stresses ROS and low pH. It has also been recently shown that inhibitors of the hypoxia-responsive two component signaling system DosRST can block hypoxia-induced INH tolerance by precluding the ability of \textit{Mtb} to sense and respond to hypoxia\textsuperscript{119}. In contrast to this mechanism, C10-treated bacteria are able to sense decreases in oxygen tension and still up-regulate the DosR regulon in hypoxic conditions, indicating that C10 sensitizes \textit{Mtb} to INH through a novel mechanism (Supplementary Table 2.S2, Supplementary Figure 2.S4). Importantly, it is unlikely that the DosRST inhibitors\textsuperscript{119} nor C10 would have been identified in traditional screens for compounds that inhibit \textit{Mtb} growth in traditional aerobic laboratory growth conditions. These new approaches to identify tolerance inhibitors expand on our possible TB treatment strategies.

The chemical library used in our screen included compounds that target chaperone-usher pilus biogenesis in \textit{Escherichia coli}\textsuperscript{188}. However, \textit{Mtb} does not encode chaperone-usher pili,
indicating that this family of compounds exhibits a different mechanism of action in *Mtb*. In addition, C10 has been shown to inhibit the CRP transcription factor in *Listeria*<sup>208,209</sup>. *Mtb* encodes two CRP homologs, CRP and Cmr<sup>210,211</sup>. The CRP regulon<sup>210</sup> and the originally defined Cmr-regulated genes<sup>211</sup> were not universally up or down-regulated during C10 treatment. Cmr has also been recently reported to regulate the DosR regulon<sup>212</sup>; however, we found that the DosR regulon was appropriately downregulated in aerated conditions and upregulated in hypoxic conditions. Instead, our data strongly support that C10 inhibits respiration and/or metabolism in *Mtb*. In particular, the assays we used cannot distinguish between direct inhibition of respiration through inhibition of ETC enzymes or indirect inhibition of respiration through disruption of central carbon metabolism, which can impact respiration by affecting NADH homeostasis<sup>33</sup>. Indeed, in addition to the up-regulation of enzymes involved in respiration, we also see up-regulation of enzymes involved in several central carbon metabolism pathways including pyrimidine biosynthesis, propionate metabolism, and amino acid metabolism, suggesting that these pathways may be affected by C10 treatment as well.

Notably, there is previous data that links energy metabolism to INH sensitivity. Compounds containing free thiols that stimulate *Mtb* respiration enhanced the bactericidal activity of INH<sup>19</sup>. However, inhibiting respiration by interfering with menaquinone biosynthesis also enhanced INH activity<sup>18</sup>. These data indicate that perturbing respiration through either stimulation or inhibition of the ETC can result in increased INH sensitivity. However, the mechanism by which perturbations in respiration affect sensitivity to INH remains unclear. Further investigations into C10 will shed light on these phenomena.

Unlike previous studies, our experiments with C10 have demonstrated that it is possible to reverse INH resistance in an *Mtb* katG mutant. These findings reveal that INH resistance is not
absolute and there are vulnerabilities that can be exploited to extend the clinical relevance of this antibiotic. In WT *Mtb*, KatG converts INH to its active form by coupling INH with NAD $^{113}$. This INH-NAD complex binds to InhA and inhibits mycolic acid synthesis, resulting in inhibition of *Mtb* growth $^{109}$. INH-NAD has also been shown to bind other proteins in *Mtb* that could be additional targets of INH $^{213}$. Our findings suggest that either 1) C10 directly or indirectly mediates the formation of the INH-NAD adduct in *katG* mutant strains of *Mtb* or 2) C10 licenses INH to inhibit another target that does not require coupling with NAD. Distinguishing between these possibilities will be the focus of future studies and will shed light on the diversity of INH activation mechanisms and INH targets in *Mtb*.

Although this manuscript is the first report that resistance to a frontline TB antibiotic can be reversed, Blondiaux *et al.* recently reported the identification of the small molecule SMARt-420, which reverses resistance to the second-line TB antibiotic ethionamide (ETH) $^{148}$. Both INH and ETH are pro-drugs that require activation in the bacteria. While the only known activator of INH is the enzyme KatG $^{113}$, ETH can be activated by multiple enzymes, including EthA and MymA, and mutations in either *ethA* or *mymA* confer a degree of resistance to ETH $^{145}$. In addition to these monoxygenases, Blondiaux *et al.* found that SMARt-420 led to up-regulation of a new ETH-activating enzyme, EthA2. SMARt-420 therefore sensitized an *Mtbe*thA* mutant to ETH by inducing expression of this alternative ETH activation enzyme. In addition to this example of reversing drug resistance in a genetic mutant, multiple efforts have focused on blocking intrinsic resistance mechanisms in *Mtb*. This includes inhibition of β-lactamases to block the intrinsic resistance of *Mtb* to β-lactam antibiotics $^{130}$ and inhibition of drug efflux pump activity or cell envelope integrity to improve drug permeability and retention $^{143,214,215}$.

Using C10 as a chemical tool, we have uncovered a strategy to alter the physiology of *Mtb*
so that the bacterium becomes susceptible to stresses it will encounter in the host as well as the frontline antibiotic INH. In particular, the unique ability of C10 to reverse INH resistance reveals that it may be possible to disarm INH resistance in the clinic, which would be of great utility to combat the global epidemic of drug resistant TB. Future studies to identify the target of C10 and elucidate the mechanism by which C10 elicits these effects will uncover novel therapeutic targets that can be exploited for future drug development.

2.6 Materials and Methods:

For more additional information on the methods used in this study that are not included in this section, see section 2.11 Supplementary Materials and Methods.

**Bacterial strains and growth conditions**

*Mtb* Erdman was inoculated from a freezer stock into Middlebrook 7H9 liquid media supplemented with 60μl/oleic acid, 5 g/l bovine serum albumin, 2 g/L dextrose, 0.003 g/L catalase (OADC), 0.5% glycerol, and 0.05% Tween 80. Actively growing *Mtb* was then inoculated into Sauton’s liquid medium (0.5 g/L KH₂PO₄, 0.5 g/L MgSO₄, 4.0 g/L L-asparagine, 6% glycerol, 0.05g/L ferric ammonium citrate, 2.0 g/L citric acid, 0.01% (w/v) ZnSO₄) and used for experiments. Viable CFU were enumerated on Middlebrook 7H10 or 7H11 agar media supplemented with OADC and 0.5% glycerol. ΔcydA *Mtb* was generated using specialized transduction as described in section 2.11 Supplementary Materials and Methods.

**Hypoxia-induced pellicle formation and tolerance assays**

Sauton’s media was inoculated with stationary phase *Mtb* at a 1:100 dilution ±C10. Culture vessels were closed tightly to restrict oxygen for 3 weeks, at which point seals on the vessels were opened, and for biofilm assays, the cultures were incubated for 2 additional weeks before pictures were
taken and/or CFU were enumerated. For tolerance assays, when hypoxic culture vessels were re-
aerated, H$_2$O$_2$ or antibiotics was pipetted into the media at the indicated concentrations. After 2
weeks of exposure to the indicated stress, bacteria were harvested from each well and CFU were
enumerated.

**Aerobic liquid media growth assays**

*Mtb* was inoculated into Sauton’s liquid medium supplemented with 0.05% Tween 80 at an OD$_{600}$
of 0.1. Unless otherwise noted, the pH of the media was adjusted to 7.0. Compounds were added
at the indicated concentrations, and when noted, OD$_{600}$ was monitored over time. Viable CFU
were enumerated at the indicated time points by plating serial dilutions of the cultures on 7H11
agar medium +OADC containing no antibiotics.

**Agar media growth assays**

For experiments in Figure 2.2D and Figure 2.3D, approximately 8x10$^7$ CFU were spread on the
surface of plates containing agar medium and incubated for 3 weeks before pictures were taken.
For the experiment in Figure 2.5B, approximately 2.5x10$^8$ CFU was spread over the surface of
plates containing Sauton’s agar medium adjusted to pH 5.5.

**Preparation of RNA and RNA-sequencing**

In aerobic conditions, RNA sequencing was performed on RNA extracted from *Mtb* cultured
aerobically in the presence of 5μM or 25μM C10 or DMSO for 48 hours. For hypoxic cultures,
*Mtb* was cultured in tightly sealed containers for 2 weeks in the presence of 50μM C10 or DMSO
before RNA was extracted and sequenced.

**Methylene blue assays**

*Mtb* was inoculated into Sauton’s medium containing 0.05% Tween 80 at an OD$_{600}$ of 0.25 and
incubated with the indicated concentration of INH, CFZ, or C10 for 4 hours in 2 mL screwcap
tubes at 37˚C in shaking conditions. Methylene blue was added to a final concentration of 0.003%, and cultures were incubated shaking for 16 additional hours before photos were taken and CFU enumerated.

**Multiplate alamar blue assays (MABAs)**

Logarithmically growing *Mtb* was inoculated into Sauton’s medium in 96 well plates with wells containing increasing concentrations of C10. Plates were incubated at 37˚C for 1 week, at which point resazurin was added and the plate was incubated at 37˚C overnight. Production of fluorescent resorufin was measured by removing samples from the plate, mixing with formalin to kill the *Mtb*, and measuring the fluorescence on a Tecan M200 Pro plate reader with excitation $\lambda_{ex} = 530$ nm and emission $\lambda_{em} = 590$ nm.

**ATP Quantification**

*Mtb* was inoculated into Sauton’s medium ± compounds at an OD$_{600}$ of 0.1 and incubated in a roller apparatus for 24 hours. An aliquot of the culture was then heat inactivated at 95˚C for 20 min and BacTiter Glo (Promega) was used to quantify ATP levels. CellROX assay to measure ROS. *Mtb* was inoculated into Sauton’s medium ± compounds at an OD$_{600}$ of 0.1 and incubated in a roller apparatus for 24 hours. CellROX Green (Thermofisher) was then used to quantify ROS.

**2.7 Acknowledgments:**

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Alice Wallenberg Foundation, and the Swedish Foundation for Strategic Research. C.L.S. and F.A. are supported by NIH R01 AI134847. K.N.F is supported by a pilot award from the Center for Women’s Infectious Disease Research at Washington University. G.A.H. is supported by a National Science Foundation Graduate Research Fellowship DGE-1745038 and the NIGMS Cell and Molecular Biology Training Grant GM007067. K.S.K and H.T. were supported by postdoctoral stipends from the JC Kempe Foundation. R.L.K. is supported by a Potts Memorial Foundation Postdoctoral Fellowship. M.E.S. was supported through Washington University BioMedRAP. This project has been funded in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Grant Number U19AI110818 to the Broad Institute.


**Competing Interests:** The authors have no competing financial interests to declare, but acknowledge that C.L.S., S.J.H., and F.A. have ownership in the company QureTech Bio AB that licenses C10 and, therefore, may financially benefit if the company is successful in marketing its product. Data and materials availability: Correspondence and requests for materials should be addressed to C.L.S. (stallings@wustl.edu) or F.A. (fredrik.almqvist@umu.se).
2.8 Figures

![Diagram of Figures]

**Figure 2.1:**  **C10 blocks hypoxia-induced tolerance to H$_2$O$_2$ and INH.** (A) The bicyclic 2-pyridone scaffold that was shared by all compounds within the screening library where compounds contained different substituents at each of the “R”-groups. (B) The chemical structure of C10. (C) *Mtb* was incubated in low oxygen in Sauton’s medium in the presence of DMSO or 50μM C10 for 3 weeks, then re-aerated and incubated for 2 additional weeks. Representative pictures from 3 independent experiments are shown. (D) *Mtb* ±50μM C10 was treated the same as the cultures in C and viable CFU/mL were enumerated at 5 weeks, n=3 and ns = not significant by unpaired t-test. (E) Schematic of stress assays. (F-G) *Mtb* was cultured in low oxygen conditions ±50μM C10 for 3 weeks, then re-aerated and treated with (F) H$_2$O$_2$ or (G) INH for an additional 2 weeks before CFU/mL were enumerated. Mean ±SEM between biological triplicates is graphed for each sample. ND=Not detected; limit of detection = 67 CFU/mL. All data in this figure was generated by KF.
Figure 2.2: C10 potentiates killing by INH and prevents the selection of INH resistant mutants. (A) WT *Mtb* was grown in aerated planktonic conditions in Sauton’s medium with 5μM or 25μM C10 ± 0.25μg/mL INH, and OD$_{600}$ was measured over 10 days. (B) The doubling time ± standard deviation (SD) of cultures in panel A was calculated between days 0-4. This time frame was chosen because the DMSO cultures were in the exponential growth phase. N/A indicates that growth was inhibited, and the calculation of doubling time did not accurately represent the data, as determined by R$^2$ value (N/A indicates R$^2$ below 0.98). (C) After 10 days of treatment, CFU/mL were enumerated from cultures in panel A. (A, C) Mean ± SEM is graphed, n=3. (D) WT *Mtb* was plated onto Sauton’s agar containing 0.5μg/mL INH ± 25μM C10. Representative pictures from 3 independent experiments are shown. (B-C) * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 by one-way ANOVA with Tukey’s test. Data in panels A, B, and C were generated by GAH and data in panel D was generated by KF.
Figure 2.3: C10 re-sensitizes the katG<sup>FS</sup> mutant to inhibition by INH. (A) katG<sup>FS</sup> Mtb was grown in Sauton’s medium with 5μM or 25μM C10 ± 0.25μg/mL INH, and OD<sub>600</sub> was measured over 10 days. (B) The doubling time of cultures in panel A was calculated between days 0-4. This time frame was chosen to be consistent with that used in Figure 2.2, however, the doubling time was similar when calculated over days 0-8. N/A indicates that growth was inhibited, and the
calculation of doubling time did not accurately represent the data, as determined by R² value (N/A indicates R² below 0.98). (C) After 10 days of treatment, CFU/mL were enumerated. (A, C) Mean ± SEM is graphed, n=3. (D) katG^{FS} Mtb was plated onto Sauton’s agar containing 0.5μg/mL INH and/or 25μM C10. Representative pictures from 3 independent experiments are shown. (E-F) Either (E) katG^{A177T} or (F) katG^{W328L} mutant Mtb was grown in Sauton’s medium with 5μM C10 ± 0.25μg/mL INH and OD\textsubscript{600} was measured over 10 days, n=2. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, ns = not significant (A, E-F) by two-way or (B-C) one-way ANOVA with Tukey’s test. Data in panels A, B, C, E, and F were generated by GAH, and data in panel D was generated by KF.
Figure 2.4: C10 inhibits respiration in *Mtb*. (A) RNA-seq was performed on *Mtb* treated with 25μM C10 for 48 hours in aerobic conditions. The functional categories based on gene annotations in Mycobrowser for the genes significantly (p\text{adj} < 0.05) up-regulated > 1.5-fold is presented in a pie chart. (B) *Mtb* was pre-treated for 4 hours with DMSO, 2.5μg/ml INH, 1μg/ml clofazimine (CFZ), or 50μM C10 before adding methylene blue for an additional 16 hours, n = 3. (C) The
viable bacteria from panel B were enumerated. (D) *Mtb* was treated with increasing concentrations of C10 and monitored for respiration in the MABA, and the IC$_{50}$ ± SD was calculated in Graphpad Prism, n = 3. (E-F) WT *Mtb* was incubated with 5 or 25μM C10 ± 0.25μg/mL INH for 24 hours and (E) ATP levels were measured in relative luminescence units (RLU) using BacTiter Glo, or (F) ROS levels were quantified by CellROX Green fluorescence in arbitrary units (AU). 1μg/mL CFZ was included as a positive control in panel F. (C-F) Mean ± SEM is graphed. * p < 0.05, **** p < 0.0001, ns = not significant by one-way ANOVA with Tukey’s test. Data in panel A was generated by GAH with assistance from DXZ, data in panels B and C were generated by KF, and data in panels D, E, and F were generated by GAH.
Figure 2.5: C10 sensitizes Mtb to acid stress. (A) WT Mtb was cultured in Sauton’s media at pH 7.0 or 5.5 in the presence of DMSO or 50μM C10 and viable bacteria were enumerated over time, n = 3. Mean ± SEM is graphed. **** p < 0.0001, ns = not significant by two-way ANOVA with Tukey’s test. (B) WT Mtb was cultured on Sauton’s agar pH 5.5 with 25μM C10 or DMSO, and pictures were taken at 43 days. For growth on pH 7.0 agar see Figure 2.2.2D. Data in panel A was generated by KF, and data in panel B was generated by GAH.
Figure 2.6: C10 potentiates killing by Q203. WT *Mtb* was cultured with 25μM C10 ± 400 nM Q203 for 15 days before enumerating surviving CFU. (A) A representative image of the culture dilutions plated to enumerate CFU is shown and (B) bacterial survival was quantified relative to DMSO-treated samples, n = 6. Mean ± SEM is graphed. ** p < 0.01, by one-way ANOVA with Tukey’s test. Data in panels A and B were generated by GAH.
2.9 Supplementary Figures

Supplementary Figure 2.S1: C10 inhibits *Mtb* biofilm formation. *Mtb* was incubated in low oxygen in Sauton’s medium in the presence of a range of C10 concentrations for 3 weeks, then re-aerated and incubated for 2 additional weeks. Representative image from at least 3 replicates is shown. Data in this figure was generated by GAH.
Supplementary Figure 2.S2: C10 does not block the development of hypoxia-induced tolerance to rifampicin (RIF), streptomycin (SM), or ethambutol (EMB). *Mtb* was cultured in low oxygen conditions ±50μM C10 for 3 weeks, then re-aerated and treated with the indicated concentration of (A) RIF or (B) SM or EMB for an additional 2 weeks before CFU/mL were enumerated. Mean ±SEM between biological triplicates is graphed for each sample. All of the data in this figure was generated by KF.
Supplementary Figure 2.S3: 

**Functional categories of genes up-regulated >1.5-fold in C10-treated cultures during hypoxia.** RNA-seq was performed on *Mtb* treated with 50μM C10 for 2 weeks in hypoxic conditions. The functional categories based on gene annotations in Mycobrowser for the genes significantly (p_{adj} < 0.05) up-regulated > 1.5-fold are presented in a pie chart. The data in this figure was generated by KF and JL.
Supplementary Figure 2.S4: C10 treatment does not inhibit the induction of the DosR regulon in hypoxic *Mtb*. (A-B) MA plots of *Mtb* transcript abundance after 2 weeks of incubation in low oxygen conditions in the (A) absence or (B) presence of 50μM C10. Each dot represents an annotated *Mtb* gene with the log$_2$ relative transcript abundance in hypoxic conditions divided by that of aerated conditions (M) plotted against the log$_2$ of its average abundance in both conditions (A). M and A values are based on data from 3 biological replicates. The dotted lines mark 3-fold differential abundance. The data in this figure was generated by KF and JL.
Supplementary Figure 2.S5: C10 inhibits growth of Mtb at high concentrations. (A-B) WT Mtb was incubated in Sauton’s media the presence of DMSO, 100μM, or 250μM C10 and (A) the OD$_{\lambda 600}$ was monitored over 10 days. (B) Viable bacteria were enumerated by plating CFU on agar media containing no drugs on Day 0 and Day 10 of the treatment, n=3. The data in this figure was generated by GAH.
Supplementary Figure 2.S6: C10 inhibits respiration in ΔcydA Mtb very similarly to WT. Either WT (n = 2) or ΔcydA (n = 4) Mtb was incubated with increasing concentrations of C10 and respiration was measured in the MABA. The data in this figure was generated by GAH.
### 2.10 Supplementary Tables

**Supplementary Table 2.S1: katG mutations in INH-resistant isolates**

katG mutations in *Mtb* colonies that grew on Sauton’s media plates containing 0.5μg/ml INH (pictured in 2C). AA stands for amino acid.

<table>
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Supplementary Table 2.S8: C10 does not block induction of the DosR regulon
RNA-seq data comparing expression of the hypoxia-responsive DosR regulon in *Mtb* cultured in hypoxic conditions for 2 weeks ±50μM C10. Fold change represents gene expression in the hypoxic samples (±50μM C10) divided by gene expression in aerobic *Mtb*.

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<th>2 week hypoxic + C10: aerobic</th>
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2.11 Supplementary Materials and Methods:

Bacterial strains and growth conditions

For each experiment, WT Mtb Erdman was inoculated from a freezer stock into Middlebrook 7H9 liquid media supplemented with 60μl/l oleic acid, 5 g/l bovine serum albumin, 2 g/L dextrose, 0.003 g/L catalase (OADC), 0.5% glycerol, and 0.05% Tween 80. Actively growing Mtb was then inoculated into Sauton’s liquid medium (0.5 g/L KH2PO4, 0.5 g/L MgSO4, 4.0 g/L L-asparagine, 6% glycerol, 0.05g/L ferric ammonium citrate, 2.0 g/L citric acid, 0.01% (w/v) ZnSO4) and used for experiments. Viable CFU were enumerated on Middlebrook 7H10 or 7H11 agar media supplemented with OADC and 0.5% glycerol. ΔcydA Mtb was generated using specialized transduction (1). The homology region upstream of cydA was amplified using Erdman genomic template DNA by RK9:

GAAGCTTGTGATACCGAACTGCCACCGCGAAATGTC and RK10:

GAGATCTCTGGCCACATAACGTCAGGG, and the downstream homology region was amplified by RK11: GTCTAGAGAGAGGCTACTAGCACCATTG and RK12:

GGGTACCGGTACCCACGTACGATCTCTG. A HindIII/SmaI fragment of the upstream region and a XbaI/KpnI fragment of the downstream region were cloned upstream and downstream of a hygromycin resistance marker (HygR). We recombined this cassette into the genome of a specialized transducing phage and transduced WT Erdman Mtb with this engineered phage. By selecting for hygromycin resistant Mtb, we identified Mtb strains that had replaced the cydA gene with HygR by homologous recombination. Successful replacement of the cydA gene was confirmed by Southern blot.

Compound Preparation
The 2-pyridone library contained a mix of compounds with a diverse substitution pattern in positions C2 and C6-C8. These were prepared by previously published methodology (2-7). C10 was prepared as previously described (8, 9). For experiments involving agar media containing C10, we developed a C10-imidazole salt (C10-IMD) with improved solubility by the following method. HPLC-purified C10 was dissolved in methanol in room temperature to which 1 equivalent of imidazole from a separately prepared imidazole (IMD) stock solution (20 mg/mL) in methanol was added. The reaction mixture was stirred at room temperature for 2 hours in a closed round-bottom flask. After 2 hours, the solvent was evaporated under vacuum and the resulting product was mixed well with 10 mL of Acetonitrile-Water (1:3). The resulting mixture was subjected to freeze drying overnight, resulting in C10-IMD, a white solid. C10-IMD was used in place of C10 in the following figures: Figure 2.2D, Figure 2.3A-F, Figure 2.4A, D-E, and Figure 2.5B. The concentration of IMD was normalized in all samples in Figure 2.2D, Figure 2.3D-F, and Figure 2.4A. In Figure 2.4E-F, the 0μM C10 ±INH samples have 25μM IMD, and in all experiments the concentration of DMSO is normalized in all samples.

**Antibiotics**

Q203 was synthesized and provided by Enamine (New Jersey). Clofazimine, Hygromycin B, isoniazid, ethambutol, streptomycin, and rifampicin were all purchased from Sigma-Aldrich.

**Hypoxia-induced pellicle formation and tolerance assays**

Sauton’s media was inoculated with stationary phase *Mtb* at a 1:100 dilution with or without C10. Culture vessels were closed tightly to restrict oxygen for 3 weeks, at which point seals on the vessels were opened, and for biofilm assays, the cultures were incubated for 2 additional weeks before pictures were taken and/or CFU were enumerated. For tolerance assays, when
hypoxic culture vessels were opened and re-aerated, H$_2$O$_2$ or antibiotics was pipetted into the media at the indicated concentrations. To enumerate viable CFU after 2 weeks of exposure to the indicated stress, bacteria were harvested from each well, centrifuged to pellet, and resuspended in 1% Tween 80 in phosphate-buffered saline (PBS). Glass beads were added to each tube and tubes were shaken overnight at room temperature to disassociate bacterial clumps. Serial dilutions were plated to enumerate viable CFU.

**katG sequencing**

To sequence the katG gene in isolated colonies from INH plates, we inoculated bacteria into liquid medium containing 0.5μg/ml INH. Genomic DNA was isolated from these cultures and katG-specific primers were used to amplify segments of the katG gene and to sequence the amplicons. Sequences were compared to the WT *Mtb* Erdman katG sequence. Primer sequences:
katG1: GCGGGTTGTGGTTGATCG, katG2: CAGCACGGCAAGGATTCC, katG3: GACAGTCAATCCCGATGC, katG4: AGCCCAAGCCCATCTG, katG5: TCGTGGATGCGGCTAGGTG. Sequencing was performed by Genewiz, South Plainfield, NJ.

**Preparation of RNA and RNA-sequencing**

cDNA libraries were prepared from total RNA using the RNAtag-Seq protocol (10). Briefly, RNA samples were fragmented, depleted of genomic DNA, and dephosphorylated prior to their ligation to barcoded adaptors. Barcoded RNAs were pooled, depleted of rRNA using RiboZero (Epicentre) and converted to Illumina cDNA libraries in 3 steps: (i) reverse transcription of the RNA primed from the ligated adapter; (ii) degradation of the RNA and ligation of a second adaptor to the single-stranded cDNA; (iii) PCR amplification of the cDNA with primers targeting the ligated adapters and carrying the full sequence of the Illumina sequencing adaptors.
The aerobic RNA libraries were sequenced on HiSeq2500 using 1x50 reads. The hypoxic RNA libraries were sequenced on HiSeq 2000 to yield 25b PE reads.

**RNA-Sequencing data analysis**

For aerobic RNA-sequencing Salmon was used in mapping-based mode to map and quantify RNA sequencing reads. A transcriptome index was created using the *M. tuberculosis* H37Rv transcriptome genome assembly ASM19595v2. Count tables were imported into R using tximport. Differential expression analysis was performed in R using DESeq2. For hypoxic RNA-sequencing, sequencing reads were aligned to the H37Rv genome (RefSeq NC_000962), and the overall fragment coverage of genomic regions corresponding to features such as open reading frames and rRNAs based on RefSeq annotations was conducted by using bioinformatic pipelines developed in house as previously described. Differential-expression analysis was conducted with DESeq. Transcript functional categorization was performed by determining the “functional category” assigned on Mycobrowser (https://mycobrowser.epfl.ch/).

**Multiplate alamar blue assays (MABAs).**

*Mtb* was inoculated from a freezer stock into 7H9+OADC and grown to mid-log phase. Logarithmically growing *Mtb* was then inoculated into Sauton’s medium in 96 well plates with wells containing increasing concentrations of C10 at an OD$_{600}$ of 0.0008, corresponding to approximately 4x10$^5$ CFU/mL in 200μL per well. Plates were incubated at 37°C for 1 week, at which point 32.5μL of a resazurin-tween mixture (8:5 ratio of 0.6mM Resazurin in 1XPBS to 20% Tween 80) was added and the plate was incubated at 37°C overnight. Production of fluorescent resorufin was measured by removing samples from the plate, mixing with formalin to kill the *Mtb*, and measuring the fluorescence on a Tecan M200 Pro plate reader with excitation...
λ_{ex} = 530 nm and emission λ_{em} = 590 nm. For each assay, media alone served as a negative control and untreated \textit{Mtb} was included as a positive control. The % inhibition was calculated as the \((\text{positive control} - \text{negative control}) - (\text{fluorescence of the sample} - \text{negative control}))/(\text{positive control} - \text{negative control}) \times 100\%.

\textbf{ATP Quantification.}

\textit{Mtb} was inoculated into Sauton’s medium ± compounds at an OD_{\lambda_{600}} of 0.1 and incubated in a roller apparatus for 24 hours. An aliquot of the culture was then heat inactivated at 95°C for 20 min and diluted 1:100. Samples were then mixed with BacTiter Glo (Promega) reagent at a 1:1 ratio, and luminescence was quantified on a Tecan M200 Pro plate reader (integration = 1 second). Luminescence was normalized to the OD_{600} of the culture to account for changes in bacterial growth.

\textbf{CellROX assay to measure ROS.}

\textit{Mtb} was inoculated into Sauton’s medium ± compounds at an OD_{\lambda_{600}} of 0.1 and incubated in a roller apparatus for 24 hours. Then 5 mL of the culture were pelleted and resuspended in 0.5 mL of media containing 10μM CellROX Green reagent (Thermofisher). Samples were incubated shaking for 37°C for 1 hour, and then washed twice with PBS containing 0.05% Tween 80 and resuspended in 4% paraformaldehyde to kill the \textit{Mtb}. Fluorescence was measured using a Tecan M200 Pro plate reader with excitation \lambda_{ex} = 485 nm and emission \lambda_{ex} = 520 nm. Fluorescence was normalized to the OD_{600} of the culture to account for changes in bacterial growth.

\textbf{Data and Statistics}

Determination of the significance of differences in measurements was performed with Prism (Graphpad Software, Inc) by calculating p values using unpaired Student’s t test (to compare two
groups) or using a one- or two-way ANOVA with Tukey’s multiple comparison test (to compare more than two groups), as indicated. To compare CFU/mL data, the data was first log$_{10}$-transformed and then a t test, one-way ANOVA, or two-way ANOVA was used to compare between groups. To determine the IC$_{50}$ values in the MABA, each replicate was graphed separately and the IC$_{50}$’s were determined for each replicate individually. Then IC$_{50}$’s were averaged and the standard deviation was determined.
Chapter 3: Inducing vulnerability to InhA inhibition restores isoniazid susceptibility in drug resistant *Mycobacterium tuberculosis*

A version of this chapter is in preparation for submission, and was deposited in BioRxiv (https://doi.org/10.1101/2023.02.06.527416) with the following co-contributors:

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Preface:

A version of this chapter comprises a manuscript in preparation for submission to a journal for publication. Most of the work in this chapter was also deposited on BioRxiv prior to publication. GAH performed all of the experiments in this chapter under the supervision of CLS, with assistance from co-authors. KC made these studies possible by performing liquid chromatography-mass spectrometry to detect INH-NAD under the supervision of GJP using *Mycobacterium tuberculosis* extracts generated by GAH. ERW assisted in troubleshooting methods to measure *de novo* mycolic acid biosynthesis by \(^{14}\text{C}\)-labeling and thin layer chromatography and provided crucial moral support during the generation of live radioactive tuberculosis in the biosafety level 3 laboratory. SS synthesized the C10 used in these experiments under the supervision of FA.
3.1 Abstract:

Of the approximately 10 million cases of *Mycobacterium tuberculosis* (*Mtb*) infections each year, over 10% are resistant to the frontline antibiotic isoniazid (INH). INH resistance is predominantly caused by mutations that decrease the activity of the bacterial enzyme KatG, which mediates conversion of the pro-drug INH to its active form INH-NAD. We previously discovered an inhibitor of *Mtb* respiration, C10, that enhances the bactericidal activity of INH, prevents the emergence of INH-resistant mutants, and re-sensitizes a collection of INH-resistant mutants to INH through an unknown mechanism. To investigate the mechanism of action of C10, we exploited the toxicity of high concentrations of C10 to select for resistant mutants. We discovered two mutations that confer resistance to the disruption of energy metabolism and allow for growth of *Mtb* in high C10 concentrations, indicating that growth inhibition by C10 is associated with inhibition of respiration. Using these mutants as well as direct inhibitors of the *Mtb* electron transport chain, we provide evidence that inhibition of energy metabolism by C10 is neither sufficient nor necessary to potentiate killing by INH. Instead, we find that C10 acts downstream of INH-NAD synthesis, causing *Mtb* to become particularly sensitive to inhibition of the INH-NAD target, InhA, without changing the concentration of INH-NAD or the activity of InhA, the two predominant mechanisms of potentiating INH. Our studies revealed that there exists a vulnerability in *Mtb* that can be exploited to render *Mtb* sensitive to otherwise subinhibitory concentrations of InhA inhibitor.

3.2 Significance:

Isoniazid (INH) is a critical frontline antibiotic to treat *Mycobacterium tuberculosis* (*Mtb*) infections. INH efficacy is limited by its sub-optimal penetration of the *Mtb* lesion, and by the
prevalence of clinical INH-resistance. We previously discovered a compound, C10, that enhances the bactericidal activity of INH, prevents the emergence of INH-resistant mutants, and re-sensitizes a set of INH-resistant mutants to INH. Resistance is typically mediated by \textit{katG} mutations that decrease the activation of INH, which is required for INH to inhibit the essential enzyme InhA. Our current work demonstrates that C10 re-sensitizes INH-resistant \textit{katG}-hypomorphs without enhancing the activation of INH. We furthermore show that C10 causes \textit{Mtb} to become particularly vulnerable to InhA inhibition without compromising InhA activity on its own. Therefore, C10 represents a novel strategy to curtail the development of INH resistance and to sensitize \textit{Mtb} to sub-lethal doses of INH, such as those achieved at the infection site.

3.3 Introduction:

The disease tuberculosis (TB), caused by \textit{Mycobacterium tuberculosis} (\textit{Mtb}), remains a global health threat. As of 2019, TB was reported to be the 13\textsuperscript{th} leading cause of death worldwide, and the current COVID-19 pandemic has exacerbated challenges in TB disease surveillance and global control efforts \textsuperscript{2,227}. A major obstacle in the treatment of \textit{Mtb} infections is that the sterilizing activity of antibiotics is slow and sometimes incomplete at the site of infection due to several contributing factors. The penetration of antibiotics into the \textit{Mtb} lesion is limited, causing \textit{Mtb} to be exposed to fluctuating and often sub-inhibitory concentrations of antibiotic, and \textit{Mtb} has the propensity to develop phenotypically drug tolerant populations in the host, which allows a population of \textit{Mtb} to persist in spite of exposure to adequate concentrations of antibiotic \textsuperscript{13,17,22,228}. Long treatment regimens are required to overcome the issues of drug penetration and bacterial drug tolerance to ultimately clear the infection. The standard of care for the treatment of active TB lasts 6 months, including a 2-month intensive phase of regular doses of isoniazid (INH), rifampicin
(RIF), pyrazinamide, and ethambutol (EMB) followed by a 4-month continuation phase of INH and RIF \(^3\). Recently, the World Health Organization approved the recommendation for a shortened 4-month treatment regimen that can be made available to some patients, which includes a 2-month intensive phase of INH, rifapentine, pyrazinamide, and moxifloxacin, followed by 2 months and 1 week of INH, rifapentine, and moxifloxacin \(^3\). As a component of the intensive and continuation phases of both longer and shorter treatment regimens, INH is a critical frontline antibiotic that is the cornerstone of our current anti-TB regimens.

The utility of INH for the treatment of \(Mtb\) infections is threatened by the emergence and prevalence of INH resistant mutant strains of \(Mtb\). An estimated 10.7% of newly infected and 27.2% of previously treated cases are INH-resistant \(^4\). INH is a prodrug, and resistance is most commonly caused by mutations in the gene \(katG\), which encodes the sole bifunctional catalase-peroxidase enzyme in \(Mtb\) that is also responsible for converting INH to its active form within the bacteria \(^4,113,229\). KatG is an oxidative defense enzyme, and its typical substrates are \(H_2O_2\) and other peroxides \(^104\). However, KatG acts on INH as a non-canonical substrate to generate a radical intermediate of INH \(^115\), which spontaneously reacts with and attaches to the abundant cofactor NAD(H), forming INH-NAD \(^112,113\). The INH-NAD adduct, the activated form of INH, inhibits the enzyme InhA \(^112,113,116\), which is the enoyl-acyl carrier protein reductase enzyme that functions in the fatty acid synthase II (FAS-II) system \(^78,111\). InhA is required for the FAS-II system to elongate the shorter fatty acids synthesized by FAS-I to generate long lipid precursors that are subsequently converted to mycolic acids (MAs) through a multi-step process. MAs are an essential structural component of the outermost layer of the \(Mtb\) cell envelope and, therefore, by inhibiting InhA, INH-NAD compromises the integrity of the \(Mtb\) cell envelope, leading to growth inhibition and death \(^67,109\).
Identifying ways to enhance the antibacterial activity of INH has the potential to greatly improve the standard of care for TB. To this end, we recently reported the identification of the bicyclic 2-pyridone compound C10 as a potentiator of INH activity in *Mtb*. At concentrations that on its own do not inhibit growth, C10 promotes killing by INH and prevents the emergence of spontaneous INH-resistant mutants. Whereas high-level resistance to INH mediated by mutations in *katG* is generally considered to render INH ineffective, we discovered that C10 was able to re-sensitize multiple INH-resistant *katG* mutants to inhibition by INH, which had previously not been thought to be possible. The ability of C10 to potentiate INH activity in both WT and INH-resistant *katG* mutant strains of *Mtb* demonstrates that there is a vulnerability in the bacteria that can be exploited to enhance the antimicrobial activity of INH and even circumvent INH resistance. Understanding the target and mechanism of action of C10 could lead to the discovery of novel therapeutic approaches that can be used in the clinic to disarm INH resistance in *katG* mutants.

In previous work, we performed RNA-sequencing on C10-treated *Mtb* and found that C10 induces a transcriptional signature consistent with inhibition of respiration. We subsequently demonstrated that C10 blocked *Mtb* oxygen consumption and decreased bacterial ATP levels, suggesting that C10 disrupts *Mtb* energy metabolism. In the current study, we aimed to determine how C10 potentiates killing by INH and whether this potentiation is linked to inhibition of *Mtb* energy metabolism. We use a combination of forward genetics and chemical biology to reveal that the inhibition of respiration by C10 is not required for C10 to potentiate INH, thereby uncoupling these two effects of C10 on *Mtb* physiology. Instead, we present evidence that C10 restores INH susceptibility in a subset of resistant mutants by enhancing the bacterial vulnerability to InhA inhibition. Our findings reveal potential strategies to improve the efficacy of INH as well
as other antibiotics that target mycolic acid metabolism in *Mtb*.

### 3.4 Results:

#### 3.4.1 Isolation of mutants that are resistant to C10

To better understand the mechanism of action of C10 (Figure 3.1A), we chose a forward genetic approach and isolated mutants that are resistant to C10, with the goal of identifying mutations in genes linked to the mechanism of action of C10. Previous studies used 25μM of C10 to disrupt *Mtb* energy homeostasis and deplete bacterial ATP. But 25μM of C10 only results in a modest decrease in *Mtb* growth. Therefore, to select for C10 resistant mutants, we first determined the concentration of C10 that was sufficient to inhibit growth of wild-type (WT) *Mtb*. By increasing the C10 concentration above 25μM, we found that C10 caused dose-dependent inhibition of *Mtb* growth both in liquid media and on agar plates (Figure 3.1B-C), consistent with our previous studies. 200μM C10 completely inhibited *Mtb* growth in both conditions, so we used this concentration to select for resistant mutants. By spreading *Mtb* on agar containing 200μM C10 and allowing these agar plates to incubate for a total of 9 weeks, we eventually observed the emergence of spontaneous resistant colonies. We isolated 11 resistant mutants and performed whole genome sequencing to identify mutations that confer the C10-resistance. We found that each resistant strain harbored one of 3 different mutations (Figure 3.1D). To probe how these mutations impact C10 sensitivity, we chose representative strains that each harbor one of these 3 mutations as the sole nucleotide change that could be identified by whole genome sequencing. Strain GHTB136 harbors an intergenic mutation between the putative S-adenosylmethionine (SAM)-methyl transferase *Rv0731c* and the *secY-adt-mapA* operon, a C to A substitution 69 bp from the start of *Rv0731c* and 92 base pairs (bp) from the start of *secY*. To determine if this mutation impacts
the expression level of the neighboring genes we performed qRT-PCR and found that the GHTB136 strain exhibited >100-fold up-regulation of the \textit{Rv0731c} gene and 2-6-fold up-regulation of the \textit{secY-akd-mapA} operon compared to WT (Figure 3.S1A). The GHTB146 strain harbors an intergenic mutation between the \textit{lpaD-glpD2} operon and the uncharacterized gene \textit{Rv3304}, an A to G substitution 12 bp from the start of the \textit{lpaD} coding region. Since \textit{lpaD} is a leaderless transcript \textsuperscript{231}, this mutation is likely located within the RNA polymerase binding region. The GHTB146 mutant exhibited 4-8-fold up-regulation of the \textit{lpaD-glpD2} operon, but no change in expression of the \textit{Rv3304} gene compared to WT (Figure 3.S1B), suggesting that this mutation enhances \textit{lpaD-glpD2} promoter activity.

The third representative strain, GHTB149, harbors a missense mutation within the putative SAM-methyl transferase \textit{Rv0830} that results in the substitution of valine for a leucine residue at position 292 (L292V).

To determine how these mutations impact the sensitivity of \textit{Mtb} to C10, we quantified the level of C10 resistance in these strains using a resazurin microplate assay. This assay takes advantage of the redox-sensitive dye resazurin, which is blue in its oxidized form but becomes reduced to the fluorescent pink product resorufin as a result of bacterial growth and metabolism. Therefore, fluorescence can be monitored as a proxy for bacterial growth and metabolism. C10 inhibits WT \textit{Mtb} in this assay with a half-maximal inhibitory concentration (IC\textsubscript{50}) of 25\textmu M (Figure 3.1E) \textsuperscript{45}. The IC\textsubscript{50} of C10 in GHTB149, which harbors the \textit{Rv0830} L292V mutation, was 59\textmu M (Figure 3.1E), a 2.4-fold increase over WT \textit{Mtb}, indicating that this mutant exhibits a low level of resistance to C10. In contrast, the C10 IC\textsubscript{50} in both GHTB136 and GHTB146 was >100\textmu M (Figure 3.1E). Therefore, while this assay confirmed that all 3 representative isolates are resistant to C10, the mutations in GHTB136 and GHTB146 confer a higher level of resistance than the GHTB149 strain.
C10 inhibits respiration and depletes ATP levels in WT *Mtb* \(^{45}\). Therefore, to directly determine how the mutations in the C10-resistant strains affected ATP-depletion by C10, we cultured WT, GHTB136, GHTB146, and GHTB149 in the presence and absence of 25\(\mu\)M C10 for 24 hours and quantified bacterial ATP levels using a luciferase-based BacTiter Glo assay (Figure 3.1F). Similar to WT, the GHTB149 strain exhibited a decrease in bacterial ATP in response to C10, suggesting that the low level of resistance conferred by the *Rv0830* L292V mutation is not sufficient to overcome the depletion of ATP by C10. In contrast, C10 did not significantly decrease the ATP levels in the GHTB136 and GHTB146 strains (Figure 3.1F), demonstrating that these mutants are resistant to the ATP-depleting effects of C10. Collectively these findings demonstrate that C10-mediated growth inhibition is linked to ATP depletion, as mutants that maintain ATP levels in the presence of C10 are able to overcome the toxicity of C10. Notably, none of the mutants exhibited an altered level of ATP at baseline (Supplementary Figure 3.S2), indicating that these strains do not overcome the effects of C10 simply by harboring increased pools of ATP. Furthermore, the C10-resistant strains were not cross-resistant to compounds known to inhibit ATP synthesis by targeting the electron transport chain (ETC), such as the ATP synthase inhibitor bedaquiline (BDQ) or the protonophore CCCP (Supplementary Figure 3.S3). Therefore, GHTB136, GHTB146, and GHTB149 are specifically resistant to the effects of C10, and the mechanisms of C10 resistance in these strains do not confer cross-resistance to respiration inhibitors in general.

### 3.4.2 C10 potentiates INH independently of its effect on energy homeostasis

Since GHTB136 and GHTB146 overcome the depletion of ATP by C10, we used these mutants to test whether disruption of energy homeostasis by C10 contributes to its ability to enhance the bactericidal activity of INH. We cultured WT *Mtb*, GHTB136, and GHTB146 in
media containing 25μM C10 and/or 0.25μg/mL INH and enumerated the colony forming units (CFU) to determine the number of viable bacteria after 10 days of treatment. Similar to our previously reported results, C10 enhanced the bactericidal effect of INH against WT *Mtb*, leading to approximately 2 orders of magnitude fewer CFU/mL after 10 days of treatment compared to INH alone (Figure 3.2A) \(^{45}\). We found that C10 still potentiated the bactericidal activity of INH against GHTB136 and GHTB146 strains to a similar or even greater extent compared to WT (Figure 3.2B-C). Therefore, C10 can enhance killing by INH in strains that maintain ATP levels during exposure to C10, demonstrating that ATP depletion is not required for C10 to potentiate INH. In support of this finding, when we examined whether the direct ATP synthase inhibitor BDQ could recapitulate the effect of C10, we found that depletion of bacterial ATP with BDQ did not potentiate killing by INH (Figure 3.2D-E). Instead, BDQ in combination with INH resulted in a 1-2-log increase in the number of viable bacteria compared to INH alone. These findings are consistent with previous reports that BDQ and other ETC inhibitors, including Q203 and CCCP, antagonize killing by INH \(^{120-122}\). Depletion of ATP is, therefore, not sufficient to potentiate INH, indicating that C10 must impart another effect on *Mtb* that is not reversed in the GHTB136 and GHTB146 mutants to elicit the increased sensitivity to INH.

### 3.4.3 Isolation of mutants that are resistant to the combination of C10 and INH

To specifically address how C10 potentiates INH activity in *Mtb*, we sought to identify genes that are required for C10 to potentiate INH by selecting for spontaneous *Mtb* mutants that can grow in the presence of C10 and INH. We had previously been unable to select for mutants that grew in the presence of 25μM C10 and 0.5μg/mL INH \(^{45}\). Therefore, we decreased the selective pressure by lowering the concentration of INH. We inoculated WT *Mtb* onto agar media containing 25μM C10 and 0.2μg/mL INH, and incubated the bacteria for 4 months at 37°C. We
isolated 3 spontaneous mutants that could grow on agar media containing 25μM C10 and 0.2μg/mL INH and performed whole genome sequencing to identify the genetic basis for resistance. Two of the mutant strains harbored large genomic deletions. One strain, GHTB089, was deleted for 27.9 kilobases (kb) of its genome (Δ2145809-2173696), which disrupted 27 annotated genes, including deleting the first 1183 bp of katG. The second isolate, GHTB092, was deleted for 38.6 kb of its genome (Δ2132215-2170824), which included the entire katG gene and 36 additional annotated genes. In contrast, the third strain harbored a single point mutation when compared to the parental WT strain, a nucleotide change in katG that results in an early stop codon at tryptophan 198. We designated this strain katG\text{W198*}.

KatG is a 740-amino acid protein with several residues that are critical for catalase-peroxidase activity, including a heme-coordinating histidine at position 270 and catalytic residues at R104, H108, and W321. The katG\text{W198*} mutation is predicted to result in truncation of over two thirds of the KatG protein, including several of these essential residues. The selection of the katG\text{W198*} mutant on media containing INH and C10 was perplexing because in our earlier study we had shown that C10 still potentiates INH in multiple INH-resistant katG mutants, including a katG mutant that harbors a frameshift mutation at amino acid 6 that results in an early stop codon (katG\text{FS6}) and a katG mutant with a single amino acid substitution of a leucine for a tryptophan at position 328 (katG\text{W328L}) \text{45}. To determine if the katG\text{W198*} mutant was truly unique from the previously studied INH-resistant katG mutants, we compared the ability of the katG\text{W198*}, katG\text{FS6}, and katG\text{W328L} mutants to grow on agar medium containing 0.5μg/mL INH and/or 25μM C10 (Figure 3.3A). As expected based on our previous data, the katG\text{FS6} and katG\text{W328L} mutants grow well in the presence of either C10 or INH alone, but are re-sensitized to INH in the presence of C10 such that their growth is inhibited on agar containing both C10 and INH together (Figure
3.3A). In contrast, the $katG^{W198*}$ mutant grew on agar containing C10 alone, INH alone, and the combination (Figure 3.3A), demonstrating that this strain is resistant to INH even in the presence of C10.

We monitored the effects of C10 and INH on the viability of each $katG$ mutant strain compared to WT by culturing the bacteria in liquid media with or without C10 and/or INH for 10 days and enumerating CFU (Fig 3B-E). Similar to our previously reported results, while the $katG^{FS6}$ mutant can grow in the presence of INH or C10 alone, 25μM C10 in combination with INH caused a significant decrease in the number of viable bacteria compared to C10 or INH alone (Figure 3.3C). Similar to the $katG^{FS6}$ mutant, the $katG^{W328L}$ mutant can grow in the presence of INH or C10 alone, but in combination with INH, 10 or 25μM C10 was able to decrease the number of CFU/mL 3-4 orders of magnitude below the inoculum (Figure 3.3D), demonstrating that C10 restores the bactericidal activity of INH against this mutant. In contrast, the CFU/mL of the $katG^{W198*}$ mutant increased from day 0 to 10 in cultures treated with C10 alone, INH alone, and the combination (Figure 3.3E). 25μM C10 significantly decreased the CFU/mL on day 10 compared to the untreated controls, but this was not enhanced by the addition of INH, indicating that C10 inhibits growth of the $katG^{W198*}$ mutant, but is unable to potentiate INH in this strain.

The ability of the $katG^{W198*}$ mutant to grow in the presence of C10 and INH could be due to resistance to the C10/INH combination or resistance to C10 specifically. To determine if the $katG^{W198*}$ mutant was resistant to C10 activity, we examined the effect of C10 treatment on ATP levels in the $katG^{FS6}$, $katG^{W328L}$, and $katG^{W198*}$ mutants by treating with 10 μM or 25 μM C10 for 24 hours and then measuring ATP levels using the luciferase-based BacTiter Glo assay. C10 treatment caused a similar dose-responsive decrease in ATP in WT Mtb and the $katG$ mutants as compared to the DMSO-treated cultures (Figure 3.3F). C10 also inhibited the $katG$ mutants to a
similar or even greater extent compared to WT in the resazurin microplate assay, with the IC\textsubscript{50} of C10 in the WT, \textit{katG}\textsuperscript{FS6}, \textit{katG}\textsuperscript{W328L}, and \textit{katG}\textsuperscript{W198*} strains being 22\textmu M, 12\textmu M, 7.4\textmu M, and 14\textmu M, respectively (Figure 3.3G). These findings demonstrate that C10 is still able to disrupt \textit{Mtb} energy homeostasis in \textit{katG}\textsuperscript{W198*} and, therefore, the loss of INH potentiation in \textit{katG}\textsuperscript{W198*} is not due to insensitivity to C10 activity.

3.4.4 KatG expression and activity is required for C10 to enhance INH inhibitory activity in \textit{Mtb}

Given the finding that C10 was able to re-sensitize both \textit{katG}\textsuperscript{FS6} and \textit{katG}\textsuperscript{W328L} mutant strains but not \textit{katG}\textsuperscript{W198*} to INH, we hypothesized that there may be a functional difference between the KatG protein variant expressed in the \textit{katG}\textsuperscript{W198*} mutant compared to \textit{katG}\textsuperscript{FS6} and \textit{katG}\textsuperscript{W328L}. To begin to investigate this possibility, we probed the expression of KatG protein in each strain by western blot using a monoclonal \textalpha-KatG antibody (Figure 3.4A). \textit{katG}\textsuperscript{W328L} \textit{Mtb} harbored similar or higher levels of KatG protein compared to WT \textit{Mtb}, whereas \textit{katG}\textsuperscript{W198*} \textit{Mtb} did not express any full length KatG protein, similar to a \textDelta katG strain. In contrast, the \textit{katG}\textsuperscript{FS6} strain expressed a low abundance protein species that was recognized by the \textalpha-KatG antibody and migrated slightly faster than the full length KatG protein expressed in WT \textit{Mtb} (Figure 3.4A). Upon closer examination of the \textit{katG} mRNA sequence, we noted that there is a putative alternative GUG start codon downstream from the early stop codon introduced by the frameshift mutation in \textit{katG}\textsuperscript{FS6} that may re-initiate translation in the correct frame beginning at codon 23. Therefore, we postulate that the faint band detected by western blot from \textit{katG}\textsuperscript{FS6} cell lysate represents low level expression of a variant KatG protein with a small N-terminal truncation (\textDelta1-22) that still contains all necessary catalytic residues (Figure 3.4A-B).

Western blot analysis indicates that the \textit{katG}\textsuperscript{FS6} and \textit{katG}\textsuperscript{W328L} mutants express KatG
protein variants that contain all necessary catalytic residues, while \( katG^{W198*} \) does not (Figure 3.4A-B). To determine if the \( katG^{FS6} \) and \( katG^{W328L} \) mutants retained KatG catalytic activity, we cultured each strain in liquid media with or without 0.25 \( \mu g/mL \) INH and monitored the growth of the culture by measuring OD\(_{600}\) over time. We found that while WT \( Mtb \) was completely inhibited by 0.25 \( \mu g/mL \) INH (Figure 3.4C), the \( katG^{FS6} \) and \( katG^{W328L} \) mutants were able to grow in the presence of INH, eventually reaching an OD\(_{600}\) over 10-fold above the inoculum (Figure 3.4D-E). However, both mutants exhibited a significant decrease in the OD\(_{600}\) compared to the untreated controls, indicating that although the \( katG^{FS6} \) and \( katG^{W328L} \) mutants exhibit decreased sensitivity to INH, some KatG activity was retained to impart this modest growth inhibition in the presence of INH (Figure 3.4D-E). In contrast, the \( katG^{W198*} \) and \( \Delta katG \) mutants were completely resistant and grew uninhibited in the presence of INH (Figure 3.4F-G), supporting that both of these mutations are \( katG \)-null alleles. Therefore, the \( katG^{FS6} \) and \( katG^{W328L} \) mutants that are re-sensitized to INH by C10 are hypomorphic for \( katG \), exhibiting decreased KatG activity leading to INH resistance, but retaining a residual level of KatG enzymatic activity (Figure 3.4D-E). In contrast, the \( katG^{W198*} \) mutant that is not re-sensitized to INH by C10 exhibits no KatG activity (Figure 3.4F), similar to a \( katG \)-null strain (Figure 3.4G). Based on these data, we hypothesized that some KatG activity is required for C10 to enhance INH sensitivity. To test this hypothesis, we examined whether C10 could sensitize a \( \Delta katG \) mutant to INH. Deletion of \( katG \) phenocopied the \( katG^{W198*} \) mutant and enabled \( Mtb \) to grow on agar and in liquid media containing both C10 and INH (Figure 3.4H-I), demonstrating that some KatG expression and activity is required for C10-mediated sensitization to INH.

### 3.4.5 C10 induces vulnerability to inhibition by INH without altering KatG activity or INH-NAD levels
Most strategies that renew sensitivity of bacterial pathogens to antibiotics do so by increasing the levels of active antibiotic. Key examples of this are β-lactamase inhibitors that prevent the degradation of β-lactam antibiotics and the recent discovery of SMARt-420, which increases the conversion of ethionamide (ETH) to its active form, ETH-NAD, in Mtb. In addition, Mtb encodes the enzyme CinA that cleaves NAD-drug adducts to promote tolerance to antibiotics like INH and ETH, highlighting that regulation of active drug concentration is a major mechanism of modulating drug efficacy. Therefore, we investigated whether C10 sensitizes Mtb to INH by promoting KatG activity to enhance the conversion of INH to INH-NAD, thus increasing the levels of INH-NAD in the cell. We monitored the effects of C10 on KatG enzymatic activity in vitro by incubating purified KatG protein with H₂O₂, a natural substrate of KatG, and monitoring KatG catalase activity as measured by the H₂O₂ degradation rate. We found that C10 did not enhance the rate or kinetics of H₂O₂ degradation by KatG in vitro, indicating that C10 did not directly affect KatG catalase activity (Supplementary Figure 3.S4A). We next tested if C10 could specifically promote INH activation by KatG by monitoring the conversion of INH to INH-NAD in vitro. Previous work showed that INH activation by KatG occurred most efficiently in the presence of Mn²⁺. Therefore, we incubated KatG protein in buffer containing Mn²⁺ with INH and NAD⁺ in the presence or absence of C10 and monitored the levels of C10, INH, NAD⁺, and INH-NAD by liquid chromatography-mass spectrometry (LC-MS). We found that while the level of C10 remained unchanged over the course of the experiment, INH and NAD⁺ were depleted from the reaction with a concomitant increase in INH-NAD, as expected since INH and NAD⁺ are consumed to produce INH-NAD (Figure 3.5A-D). C10 did not impact the rate or level of INH-NAD produced in these conditions. In addition, although we were able to detect the interaction between KatG and INH in vitro (Supplementary Figure 3.S4B), we were unable to detect a direct
interaction between C10 and KatG using a thermal shift assay (Supplementary Figure 3.S4C),
together supporting that C10 does not directly bind KatG or promote its enzymatic activity in vitro.

To determine if C10 affects KatG activity within the bacterium, we examined whether C10 promotes KatG expression, which could explain how C10 sensitizes WT and katG hypomorph strains to INH but does not sensitize katG-null strains. We treated both WT and katG<sup>FS6</sup> Mtb with C10 for 3 days and collected whole cell lysate to perform a western blot analysis for KatG. We found that C10 did not increase the protein levels of KatG in these conditions (Figure 3.5E). We also monitored the effect of C10 on KatG catalase activity in Mtb by treating the bacteria with C10 for 6 days, collecting whole cell lysate, and measuring the H<sub>2</sub>O<sub>2</sub> degradation rate of the lysate. The ΔkatG mutant exhibited no H<sub>2</sub>O<sub>2</sub> degradation in this assay, demonstrating that the assay is specific for KatG (Figure 3.5F). While the lysate from katG<sup>FS6</sup> Mtb had significantly more activity than the ΔkatG mutant, this strain exhibited a greater than 10-fold reduction in catalase activity compared to WT, consistent with the katG<sup>FS6</sup> strain being hypomorphic for katG. We found that C10 did not change the H<sub>2</sub>O<sub>2</sub> degradation rate of WT or katG<sup>FS6</sup> Mtb (Figure 3.5F), confirming that C10 treatment does not enhance the expression or activity level of KatG within Mtb. However, this finding did not rule out whether C10 could enhance the levels of INH-NAD within the bacteria without affecting KatG activity. For example, deletion of cinA, which encodes an INH-NAD degrading enzyme, leads to elevated INH-NAD accumulation and increased INH sensitivity by decreasing the rate of INH-NAD degradation in Mtb<sup>126</sup>. To determine if C10 promotes INH-NAD accumulation in Mtb, we cultured WT, katG<sup>FS6</sup>, and katG<sup>W328L</sup> Mtb with and without C10 and/or INH for 3 days, collected aqueous metabolite extracts from the bacteria, and measured the amount of activated INH-NAD in the bacterial extract using LC-MS. We included the katG-null mutants katG<sup>W198*</sup> and ΔkatG as controls. We found that upon treatment with INH alone, WT, katG<sup>FS6</sup>, and
$katG^{W328L}$ strains all accumulated INH-NAD, and the $katG^{F56}$ and $katG^{W328L}$ mutants produced significantly decreased levels of INH-NAD compared to WT $Mtb$, confirming that these strains are indeed hypomorphic for $katG$ (Figure 3.5G). As expected, the $katG$-null mutants $katG^{W198*}$ and $\Delta katG$ were deficient in INH-NAD synthesis (Figure 3.5G). C10 did not significantly increase the amount of INH-NAD in any of the strains tested (Figure 3.5G) and, therefore, does not affect INH-NAD synthesis or degradation. This data demonstrates that C10 sensitizes $Mtb$ to INH through a novel mechanism of action without impacting the levels of INH-NAD in the bacteria.

### 3.4.6 C10 sensitizes $Mtb$ to direct inhibition of InhA without inhibiting InhA itself

Our data indicates that although the effect of C10 on INH sensitivity relies on KatG activity, C10 does not increase the expression or enzymatic activity of KatG. Based on these findings, we hypothesize that C10 acts downstream of KatG to enhance the antibacterial activity of INH-NAD after it is produced, such that even the $katG$ hypomorphic strains that produce lower levels of INH-NAD become inhibited by this lower concentration in the presence of C10. INH-NAD inhibits $Mtb$ growth by binding the NADH binding pocket in the enoyl-acyl carrier protein reductase InhA, leading to inhibition of mycolic acid biosynthesis $^{54,56,109,112,116}$. Therefore, it is possible that C10 renders $Mtb$ more sensitive to inhibition of InhA.

In addition to the frontline antibiotic INH, a second line antibiotic ethionamide (ETH) also inhibits $Mtb$ by targeting InhA. ETH is modified by one of 3 activating enzymes, EthA, EthA2, or MymA $^{144,145,148}$, generating an ETH-NAD adduct that inhibits InhA in a similar mechanism to INH-NAD. Under normal culture conditions, EthA2 is not expressed $^{148}$, and ETH is primarily activated by EthA or MymA $^{144,145}$. Genetic disruption of either ethA or mymA confers a 5.7- or 4.6-fold increase in the IC$_{50}$ of ETH respectively, and a double mutant is $>$20-fold more resistant
Therefore, EthA and MymA together comprise the major ETH-activating enzymes. To test if C10 sensitizes *Mtb* to InhA inhibition, we cultured WT *Mtb* with C10 and/or ETH and quantified the surviving CFU after 10 days of treatment (Supplementary Figure 3.S5A). Curiously, we found that C10 did not enhance the bactericidal activity of ETH (Supplementary Figure 3.S5A), despite the highly similar mechanism of action to INH. Since the activation of ETH is dependent on expression of *ethA*, *ethA2*, and *mymA*, we examined whether C10 impacted the expression of these genes in our RNA-sequencing dataset. We found that after 48 hours of exposure to 25μM C10, *Mtb* exhibits significant >2.5-fold down-regulation of *mymA*, whereas *ethA* was expressed at 1.2-fold and *ethA2* was expressed at 1.3-fold relative to the untreated control (Supplementary Figure 3.S5B). Therefore, while C10 does not enhance the bactericidal activity of ETH, C10 significantly decreases the expression of one of the primary ETH-activating enzymes, which could lead to decreased ETH activation in the presence of C10, although this remains to be tested directly. Still, the possible effect of C10 on ETH activation could obscure the potentiating effects of C10 on InhA inhibition by ETH-NAD.

To test if C10 enhances the bacterial sensitivity to InhA inhibition, we used the direct InhA inhibitor NITD-916, which does not require any known enzyme for activation. We hoped that using NITD-916 to directly inhibit InhA would avoid the confounding effect of altered drug activation in the presence of C10. We cultured WT *Mtb* with C10 and/or NITD-916 and quantified the surviving CFU after 10 days of treatment. Similar to the effect of C10 on INH sensitivity, we found that treating *Mtb* with C10 in combination with NITD-916 caused a significant decrease in the number of surviving bacteria compared to NITD-916 alone, leading to an additional 2-3 orders of magnitude decrease in survival (Figure 3.6A). Therefore, C10 increases the bacterial sensitivity to direct InhA inhibition.
One possible mechanism by which C10 could enhance the susceptibility of *Mtb* to InhA inhibition would be to decrease the expression or activity of InhA, thereby lowering the amount of INH-NAD or NITD-916 required to inhibit this target\(^{127}\). When we analyzed our previously published RNA-sequencing data from C10-treated cultures, we found that after treatment with 25µM C10 for 48 hours, *inhA* was expressed at 1.1-fold relative to the untreated control \(^{45}\), demonstrating that C10 does not decrease *inhA* expression at the transcriptional level. We next examined whether C10 compromises InhA activity by culturing *Mtb* with \(^{14}\)C-acetate and measuring *de novo* mycolic acid biosynthesis by thin layer chromatography (TLC). In the final steps of mycolic acid biosynthesis, the mycolic acid moiety is coupled to trehalose to form trehalose monomycolate (TMM), which can be transported out of the cell to the envelope\(^{93,95}\). The mycolic acid can then be trans-esterified from TMM to the arabinose moieties of arabinogalactan to form the inner leaflet of the mycolic acid layer that is covalently attached to the underlying cell wall layers \(^{64,97,98}\). Alternatively, the mycolic acid can be trans-esterified to a second TMM molecule to form trehalose dimycolate (TDM) \(^{96}\), which comprises a major component of the freely associated lipid layer that is intercalated within the covalently attached mycolic acids. Free mycolic acid, TMM, and TDM are the primary forms of mycolic acid that are not covalently linked to the cell wall, making them readily extractable and easy to separate by TLC, so we focused our analysis on these mycolic acid species as a read-out for *de novo* biosynthesis. Cultures of WT and *katG*\(^{W328L}\) *Mtb* were labeled with \(^{14}\)C-acetate for 20 hours in the presence or absence of C10 and/or INH before extracting whole cell lipids and monitoring the incorporation of the \(^{14}\)C into mycolic acids by TLC (Figure 3.6B-D; Supplementary Figure 3.S6). As standards, we used TDM purified from H37Ra (Invivogen), free mycolic acid saponified and extracted from the H37Ra TDM, the representative fatty acid oleic acid, and free \(^{14}\)C-acetate. Although we did not have a standard for
TMM, we identified a band that we predict corresponds to TMM in our samples since it migrated slower than TDM due to the overall increased polarity and based on the migration pattern reported in published studies \(^{234}\).

As expected, INH treatment of WT \textit{Mtb} decreased the intensity of bands corresponding to free mycolic acid, TDM, and TMM (Figure 3.6B-D; Supplementary Figure 3.S6A-D), and significantly increased the intensity of a band that co-migrates with oleic acid (Supplementary Figure 3.S6E). We believe this oleic acid co-migrating band represents FAS-I-generated fatty acids that serve as substrates for mycolic acid biosynthesis, thus, it is not surprising that inhibition of InhA would lead to their accumulation \(^{67,78,111}\). In contrast, INH treatment of \textit{katG}^{W328L} \textit{Mtb} did not affect the levels of free mycolic acids, TDM, or TMM over the 20 hour period, supporting less efficient InhA inhibition due to the mutation in \textit{katG} and decreased INH-NAD levels (Figure 3.6B-D; Supplementary Figure 3.S6). Addition of C10 on its own or in combination with INH did not decrease the synthesis of free mycolic acid, TDM, or TMM in the bacteria (Figure 3.6B-D; Supplementary Figure 3.S6), demonstrating that C10 does not inhibit InhA within the bacteria. While these studies do not rule out that C10 impacts the mycobacterial cell envelope in other ways, our data demonstrate that C10 potentiates the bactericidal effect of InhA inhibition without inhibiting InhA itself or enhancing the ability of INH-NAD to inhibit InhA in \textit{Mtb}.

### 3.4.7 C10 antagonizes arabinogalactan biosynthesis inhibitors but sensitizes \textit{Mtb} to other cell wall targeting antibiotics.

Since C10 enhances the bactericidal effect of direct InhA inhibition without inhibiting mycolic acid biosynthesis itself, we wondered whether the potentiating effects of C10 are specific for InhA inhibitors, or if C10 generally sensitizes \textit{Mtb} to cell envelope targeting antibiotics. The mycolic acid layer of the cell envelope is tethered to the long, branched polysaccharide
arabinogalactan, which is linked to the mesh of peptidoglycan that encases the bacterial cell. Each of these layers of the \textit{Mtb} cell wall is essential for \textit{Mtb} to grow and survive. Indeed one of the other frontline antibiotics used to treat \textit{Mtb} infections is EMB, which inhibits the arabinosyltransferases that are required to synthesize the arabinogalactan layer \textsuperscript{235,236}. The small molecule BTZ043 is in clinical development as an inhibitor of the enzyme DprE1, which is required to epimerize decaprenyl-phospho-ribose to decaprenyl-phospho-arabinose, an essential precursor to arabinogalactan synthesis \textsuperscript{237}. Furthermore, the combination of the $\beta$-lactam peptidoglycan biosynthesis inhibitor meropenem and $\beta$-lactamase inhibitor clavulanate has been shown to inhibit \textit{Mtb}, and there are recent clinical trials evaluating the efficacy of this drug combination for the treatment of TB patients \textsuperscript{130,139}. The antibiotic delamanid was recently approved for the treatment of TB in several countries world-wide. While the precise mechanism of action by which delamanid inhibits \textit{Mtb} is unclear, delamanid belongs to a class of antibiotics that have been shown to inhibit \textit{Mtb} respiration by production of the ETC-poisoning metabolite nitric oxide and to block the synthesis of some classes of mycolic acids \textsuperscript{238–240}. Unlike INH, delamanid does not inhibit the synthesis of all mycolic acids \textsuperscript{238}, suggesting that it is not an InhA inhibitor.

We examined whether C10 could sensitize \textit{Mtb} to these other cell wall targeting antibiotics similarly to INH and NITD-916 (Figure 3.7). In contrast to the potentiating effect of C10 on INH, we found that C10 significantly decreased the bactericidal effect of the arabinogalactan inhibitors EMB and BTZ043, allowing more bacteria to survive in the presence of these inhibitors (Figure 3.7). This antagonistic effect of C10 on arabinogalactan targeting antibiotics indicates that C10 does not generally enhance the sensitivity of \textit{Mtb} to all cell wall inhibitors. Previously published studies using \textit{Mycobacterium bovis} demonstrated that depleting the bacterial ATP levels with the ATP synthase inhibitor BDQ or the proton uncoupling antibiotic CCCP antagonizes the
bactericidal effect of EMB, similar to what has been reported for INH. Therefore, it is likely that C10 antagonizes the bactericidal activity of EMB and BTZ043 as a result of its ATP-depleting effect. In contrast, we found that C10 significantly enhanced killing by the combination of meropenem and clavulanate (Figure 3.7), indicating that C10 sensitizes Mtb to peptidoglycan synthesis inhibition.

Additionally, C10 enhanced bacterial killing in the presence of delamanid (Figure 3.7). The effect of C10 on delamanid sensitivity could be due to the perturbation in the mycolic acid composition caused by delamanid. Alternatively, since other inhibitors in the same class of compounds as delamanid have been shown to inhibit the Mtb ETC by generating nitric oxide, it is possible that C10 sensitizes Mtb to the inhibition of energy homeostasis caused by delamanid. We previously showed that C10 enhances the bactericidal effect of other antibiotics that target Mtb bioenergetics, such as the ETC inhibitor Q203. Consistent with this model, we found that delamanid on its own depletes bacterial ATP levels (Supplementary Figure 3.8), indicating that it is also compromises Mtb energy homeostasis.

As a control, we examined whether C10 potentiated the bactericidal activity of the RNA polymerase inhibitor RIF, since RIF does not directly target the mycobacterial cell wall. We found that C10 did not significantly alter the bacterial survival during treatment with RIF (Figure 3.7), demonstrating that C10 does not function non-specifically to enhance the bacterial sensitivity to any antibiotic. Specifically, our data shows that C10 sensitizes Mtb to killing by the InhA inhibitors INH and NITD-916, the peptidoglycan synthesis inhibitor cocktail meropenem/clavulanate, and the antibiotic delamanid, which impacts both energy homeostasis and the cell wall mycolic acid composition. These findings demonstrate that C10 can sensitize Mtb to certain cell wall targeting antibiotics, in particular those that target the mycolic acid layer or peptidoglycan synthesis, but
C10 does not induce sensitivity to arabinogalactan biosynthesis inhibitors. Therefore, while C10 does not induce broad sensitivity to cell envelope stress in general, exposing \textit{Mtb} to C10 significantly induces vulnerability to multiple cell wall targeting antibiotics.

3.5 Discussion:

Together, our findings show that C10 sensitizes \textit{Mtb} to INH without changing the concentration of INH-NAD or decreasing the activity of its target InhA, which are the two predominant mechanisms of potentiating INH reported in the literature thus far \cite{20,125–127}. Preventing DosR-induced growth arrest and increasing \textit{Mtb} respiration has also been shown to enhance the bactericidal effect of INH \cite{19,119}. However, C10 does not inhibit DosR signaling and decreases \textit{Mtb} respiration \cite{45}, indicating that C10 is also not working through these mechanisms.

In addition, our studies herein uncouple the effect of C10 on ATP production from the sensitization to INH. Instead, our data supports a model where C10 potentiates INH by making \textit{Mtb} particularly vulnerable to the depletion of cell wall mycolic acids, even in the INH-resistant \textit{katG} hypomorphs that accumulate a significantly lower concentration of INH-NAD. Mycolic acid biosynthesis is essential in mycobacteria and not conserved in non-actinobacteria or eukaryotes, making this process a very attractive target for the development of specific antimycobacterials to treat TB. This is exemplified by INH, which remains a cornerstone of TB treatment, as well as the recent approval of mycolic acid inhibitors delamanid and pretomanid for the treatment of TB and the ongoing development of direct InhA inhibitors, such as NITD-916 \cite{233,238,240}. In contrast to the potentiation strategies that increase the INH-NAD concentration or decrease InhA expression, which would be specific for INH or InhA inhibitors, respectively, it remains possible that C10 could sensitize \textit{Mtb} more generally to inhibitors of mycolic acid biosynthesis. Furthermore, our finding that C10 also
enhances the bactericidal effect of meropenem/clavulanate and delamanid indicates that the potentiating effect of C10 could be applied to the use of additional cell envelope targeting antibiotics that are currently being evaluated or even used in the clinic as second-line therapies. However, C10 does not enhance the activity of ETH and actually antagonizes killing by arabinogalactan biosynthesis inhibitors, suggesting that not all cell wall targeting antibiotics would be candidates for combination therapy with C10.

The clinical utility of INH is currently being threatened by the increasing rates of INH-resistant TB cases. While resistance to INH can occur through multiple mechanisms, the predominant cause of INH resistance is mutation of katG, which accounts for an estimated 78.6% of INH resistant strains. Clinically, mutations in katG are considered to confer a high level of resistance, often necessitating the use of an alternative treatment regimen. However, the overwhelming majority of these katG mutations are not null alleles. The most common resistance variant is an S315T amino acid substitution in KatG that decreases the enzyme’s affinity for INH. This S315T mutation and other single amino acid substitutions that are commonly identified in resistant isolates significantly impair the ability of KatG to activate INH, but these mutations do not completely abolish INH-NAD synthesis by the KatG enzyme in vitro. The complete inactivation of KatG is likely detrimental to Mtb survival in the host due to the role of KatG in the oxidative stress response, which could explain why the majority of INH resistant clinical isolates harbor single amino acid substitutions as opposed to more deleterious mutations. We found that C10 selectively potentiates killing by INH in katG mutants that express katG and retain its enzymatic activity, revealing that it is possible to rescue the utility of INH against clinically relevant INH-resistant isolates that retain a low level of KatG activity.

INH resistance can also occur upon mutation of the inhA locus that either results in
increased expression of \textit{inhA} or in expression of an InhA variant that has decreased binding to INH-NAD. An estimated 6.8\% of INH-resistant clinical isolates harbor mutations in the \textit{inhA} promoter without a concurrent \textit{katG} mutation \cite{4}. Since these mutations confer a low level of INH resistance, patients infected with these strains can be treated with a higher dose of INH to successfully clear the infection \cite{247}. Although it has not been directly tested, we anticipate that C10 would potentiate INH efficacy against \textit{inhA} promoter mutants by inducing sensitivity to even low level inhibition of mycolic acid synthesis in these strains.

In addition to resistance, INH efficacy can be limited by its sub-optimal ability to reach \textit{Mtb} at the site of infection. For instance, a clinical study that quantified the distribution of antibiotic across lung lesions from TB patients within 24 hours of dosing showed that approximately 35\% of lesions harbored sub-inhibitory concentrations of INH, likely due to a combination of drug diffusion and host metabolism \cite{13}. Therefore, the \textit{Mtb} within these lesions likely experiences fluctuating concentrations of antibiotic that can be sub-inhibitory. By making \textit{Mtb} more sensitive to InhA inhibition, C10 represents a possible strategy to sensitize \textit{Mtb} to even sub-inhibitory concentrations of antibiotic, suggesting that in addition to circumventing INH resistance, C10 could enhance the efficacy of INH at the site of infection, although this remains to be tested. While the precise mechanism by which C10 induces sensitivity to InhA inhibition remains unclear, by deciphering how C10 promotes susceptibility to INH and NITD-916, we could reveal cryptic vulnerabilities in \textit{Mtb} that can be exploited to enhance our current antimicrobial regimen.

\section*{3.6 Materials and Methods}

\textbf{Bacterial strains and growth conditions}
Mtbd Erdman strains (Table 3.1) were inoculated from a freezer stock into Middlebrook 7H9 liquid medium supplemented with 60 μL/L oleic acid, 5 g/L BSA, 2 g/L dextrose, 0.003 g/L catalase (OADC), 0.5% glycerol, and 0.05% Tween 80 and cultured at 37°C. Actively growing Mtbd was then inoculated into Sauton’s liquid medium (0.5 g/L KH₂PO₄, 0.5 g/L MgSO₄, 4.0 g/L L-asparagine, 6% glycerol, 0.05 g/L ferric ammonium citrate, 2.0 g/L citric acid, and 0.01% (wt/vol) ZnSO₄, pH 7.0) supplemented with 0.05% Tween 80 and grown to late-log phase before use in growth curve and survival experiments. The ΔkatG strain was generated using specialized transduction with the temperature sensitive phage phAE87 engineered to harbor sequence homologous to regions upstream (Erdman nucleotides 2144027-2144776) and downstream (Erdman nucleotides 2146989-2147705) of katG and selected on 50μg/mL hygromycin as previously described. For Mtbd growth and survival experiments, Mtbd was inoculated into roller bottles containing Sauton’s medium supplemented with Tween 80 at a starting OD₆₀₀ of 0.1, and growth was measured by OD₆₀₀ and survival was monitored as CFU/mL. Viable CFU from bacterial cultures were enumerated on Middlebrook 7H11 agar medium supplemented with OADC and 0.5% glycerol and plates were incubated at 37°C with 5% CO₂ for 2-3 weeks. To select for mutants resistant to both C10 and INH, the equivalent of 0.5mL of OD₆₀₀=1.0 of Mtbd growing in 7H9+OADC media was spread on Sauton’s agar containing 25μM C10 and 0.2μg/mL INH and incubated at 37°C and 5% CO₂ for 4 months. Isolated colonies were passaged on agar containing 25μM C10 and 0.2μg/mL INH to ensure that they were resistant before performing whole genome sequencing. For agar growth assays, 0.003 g/L bovine catalase was included in the Sauton’s agar, and the equivalent of 2.5mL of OD₆₀₀=1.0 of Mtbd was spread on the agar surface to enhance the reproducibility of Mtbd growth on the agar plates.

Whole genome sequencing
Genomic DNA was isolated using cetyltrimethylammonium bromide-lysozyme lysis, followed by chloroform-isoamyl alcohol extraction and isopropanol precipitation, as previously described \textsuperscript{248}. Whole-genome sequencing was performed by use of an Illumina NovaSeq 6000. The identification of single nucleotide polymorphisms was done using SeqMan NGen software (DNASTAR). The genomes were assembled and compared to the genomic DNA from the WT parental control strain, using Integrative Genome Viewer to visualize and confirm changes within regions of interest \textsuperscript{249}. Whole genome sequencing data will be deposited in the Sequence Read Archive.

**Preparation of compounds**

C10 was synthesized using previously described methods \textsuperscript{185,250} and prepared as an imidazole salt as described previously \textsuperscript{45}. Stocks of C10-imidazole were resuspended in DMSO. INH (Sigma) was dissolved in water, and NITD-916 (Sigma) was dissolved in DMSO. In all experiments, the concentration of both DMSO and imidazole were normalized across all samples to ensure that any differences were due to the effect of the indicated compounds and not due to DMSO or imidazole.

**Detection of ATP**

*Mtb* growing in Sauton’s media was treated with the indicated concentration of C10 for 24 hours before the OD\textsubscript{600} of each culture was measured and samples were removed. *Mtb* samples were inactivated at >95°C for 20 min and stored at -20°C until analyzing the ATP levels using the BacTiter Glo assay (Promega) as previously described \textsuperscript{45}. Samples were diluted 1:10, then mixed 1:1 with the BacTiter Glo reagent in a white, opaque 96-well dish, and the luminescence was read on a Synergy HT plate reader with a 1 second integration. The relative luminescence units (RLU) were calculated by subtracting the luminescence of a media only control from the luminescence value of each sample. The RLU/OD\textsubscript{600} was determined to account for differences in bacterial density, and the fold-change in each sample was calculated relative to the average of the 0μM C10
control from that experiment to facilitate combining of multiple experiments onto a single graph.

**Resazurin assay**

Logarithmically growing *Mtb* was inoculated into Sauton’s medium in 96 well plates with wells containing increasing concentrations of C10. *M. tuberculosis* was inoculated at an OD\textsubscript{600} of 0.0025 in 200 μL per well. The plates were incubated at 37°C in 5% CO\textsubscript{2} for 1 week, at which point 32.5 μL of a mixture containing an 8:5 ratio of 0.6 mM resazurin (Sigma) dissolved in 1X phosphate-buffered saline to 20% Tween 80 was added, and the production of fluorescent resorufin was measured on a Synergy HT plate reader with excitation λ\textsubscript{ex} = 530 nm and emission λ\textsubscript{em} = 590 nm after incubation at 37°C in 5% CO\textsubscript{2} overnight. For each assay, medium alone served as a negative control, and untreated *Mtb* was included as a positive control. The percent inhibition was calculated as the \{[(fluorescence of the positive control − fluorescence of the negative control) − (fluorescence of the sample − fluorescence of the negative control)]/(fluorescence of the positive control − fluorescence of the negative control)\} X 100.

**Quantitative reverse transcription PCR (qRT-PCR)**

RNA was isolated from WT, GHTB136, and GHTB146 *Mtb* growing in Sauton’s medium using Trizol, and purified by chloroform extraction followed by isopropanol precipitation. cDNA was prepared using the SuperScript III first strand synthesis kit (Invitrogen), and qPCR was performed using a SYBR green kit (Bio-Rad) with gene-specific primers (Table 3.2) on a CFX96 Real Time System (Bio Rad). The relative expression of genes was calculated using the 2\(^{-\Delta\Delta Ct}\) method, with *sigA* serving as an internal reference control gene.

**Western blot for KatG protein**

*Mtb* samples were pelleted and resuspended in buffer containing 10mM sodium phosphate pH 8.0, 150mM NaCl, 2mM ETDA, 1mM PMSF, 0.1% NP-40, and a 1X protease inhibitor cocktail.
(Roche), then lysed by bead beating, and filtered twice through a 0.22μm Spin-X column (Costar) to remove unlysed *Mtb*. SDS-polyacrylamide gel electrophoresis was performed and samples were transferred to a nitrocellulose membrane, after which KatG was detected using a mouse monoclonal α-KatG antibody used at 1:500 dilution (clone IT-57; BEI Resources). Either CarD or RpoB served as a loading control, using a mouse monoclonal α-CarD antibody at 1:2000 dilution (clone 10F05; Memorial Sloan-Kettering Cancer Center) or a mouse monoclonal α-RpoB antibody at 1:1000 dilution (clone 8RB13; Neoclon). The membrane was probed with a goat anti-mouse antibody conjugated to horseradish peroxidase and bands were visualized using the Western Lighting Plus-ECL reagent (Perkin Elmer). When performing the KatG expression analysis in response to C10 treatment, the amount of protein in each sample was measured by BCA (Pierce) and the amount of protein loaded in each lane was normalized to 67ng to facilitate comparisons between samples.

**Purification of *Mtb* KatG protein**

The *Mtb* katG coding region was cloned into *N*otI and *E*coRI sites in the pGEX-6P-1 expression vector to translationally fuse glutathione-S-transferase to the N-terminus of the KatG protein and the expression of the fusion protein was induced in logarithmically growing *E. coli* BL21-DE3 cells by treating 1L of cells with 0.1mM IPTG for 4 hours. Cells were pelleted, resuspended in 20mL 1X PBS containing a 1X protease inhibitor cocktail, and lysed twice in a cell disruptor. Lysate was treated with 9U/mL benzonase (Sigma) and clarified by centrifugation. GST-KatG was purified from the supernatant by incubating lysate overnight with glutathione agarose resin (Goldbio), washed with 300mL 1X PBS, and eluted from the resin by cleaving the KatG protein from the GST tag using PreScission protease in buffer containing 50mM Tris-HCl pH 7.0, 150mM NaCl, 1mM EDTA, and 1mM DTT.
**Thermal shift assay on KatG protein**

The melting temperature ($T_m$) of KatG in the presence of C10 or INH was determined by differential scanning fluorimetry. Purified *Mtb* KatG protein (1.1μM) was incubated with INH or C10 in 50mM potassium phosphate buffer pH 7.0 containing 50μM MnCl$_2$ before mixing samples with SYPRO$^\text{TM}$ Orange (ThermoFisher) at a final concentration of 1X in a 96-well PCR plate. The plate was incubated for 5 seconds at increasing temperatures in 0.5°C increments from 10-95°C and fluorescence was monitored over time in the HEX channel on a CFX96 Real Time System (Bio Rad). The $T_m$ was calculated by fitting curves with a Boltzmann sigmoidal equation in GraphPad Prism, and the $\Delta T_m$ was calculated as the difference between the $T_m$ of each sample and the average of the untreated control samples.

**Hydrogen peroxide degradation assay**

The catalase activity of either purified KatG or *Mtb* cell lysates was measured in a UV/Vis spectrophotometer using quartz cuvettes. For *in vitro* assays of purified KatG activity, 1.9mL of 50mM potassium phosphate buffer pH 7.0 containing 25nM purified KatG was incubated with or without C10 and/or INH at 30°C for 5min and the sample was used to blank the spectrophotometer before the indicated concentration of H$_2$O$_2$ was added to initiate the reaction, bringing the final volume of the reaction to 2mL. For samples containing *Mtb* lysate, the samples were bead beat as described above and the protein concentration in the lysate was measured by BCA (Pierce). Samples were normalized such that 25μg of total cell protein was present in each assay sample in 1.9mL of 50mM potassium phosphate buffer pH 7.0, and the reactions were initiated with the addition of H$_2$O$_2$ to a concentration of 5mM in 2mL final volume. The absorbance at 240nm was read every 10 seconds for 2 min, and the negative slope of the curve was used to calculate the rate of H$_2$O$_2$ degradation. The absorbance was converted to molarity using the extinction coefficient.
for H₂O₂, ε₂₄₀=43.6M⁻¹cm⁻¹.

**INH-activation assay and detection of INH-NAD in *Mtb***

The activation of INH was monitored *in vitro* using 200nM purified KatG protein in 50mM potassium phosphate buffer pH 7.0, 50μM NAD⁺, 50μM INH, 50μM MnCl₂, and the indicated concentration of C10 in a final volume of 1mL. At the indicated time points, 100μL were removed from the reaction and inactivated in 100μL ice cold methanol and stored at -20°C before ultra-high performance liquid chromatography/mass spectrometry (UPLC/MS). To monitor INH activation in live *Mtb*, 50mL cultures of *Mtb* in Sauton’s media without Tween 80 were treated with the indicated concentration of C10 and/or INH for 3 days. To extract polar metabolites, the cultures were pelleted, washed twice in H₂O, and resuspended in 1.5mL of 2:1 chloroform:methanol in glass conicals. Samples were kept on ice and vortexed each for 1 minute in 20 second intervals, and stored at 4°C overnight. 375μL of H₂O was added, the samples were vortexed for 1 minute in 20 second intervals, keeping the samples on ice. Samples were incubated at room temperature for 1 hour with constant agitation, and then centrifuged for 10 minutes at 1000 RPM. The top aqueous layer was transferred to a fresh 1.5mL microcentrifuge tube, stored overnight at -20°C, centrifuged for 5 minutes to pellet any insoluble material, and the supernatant was transferred to a fresh tube. INH-NAD was detected using methods similar to those previously described [117,125,126] with some modifications. UPLC/MS was performed with an Agilent 1290 Infinity UHPLC system interfaced with an Agilent 6530 QTOF mass spectrometer. Hydrophilic interaction liquid chromatography (HILIC) analysis was performed by using a HILICON iHILIC-(P) Classic column with the following specifications: 100 mm x 2.1 mm, 5 μm. Mobile-phase solvents were composed of A = 20 mM ammonium bicarbonate, 0.1% ammonium hydroxide (adjusted to pH 9.2) and 2.5 μM medronic acid in water:acetonitrile (95:5) and B = 2.5 μM medronic acid in acetonitrile:water.
(95:5). The column compartment was maintained at 45°C for all experiments. The following linear gradient was applied at a flow rate of 250 μL/min: 0-1 min: 90% B, 1-12 min: 90-35% B, 12-12.5 min: 35-20% B, 12.5-14.5 min: 20% B. The column was re-equilibrated with 20 column volumes of 90% B. The injection volume was 2 μL for all experiments. Data were acquired in both positive and negative ion modes. The mass/charge (m/z) and retention times (RT) of the compounds were as follows: INH m/z=138.066188, RT=1.87 min; C10 m/z=378.11584, 0.92 min; NAD+ m/z=664.116399, RT=6.12 min; INH-NAD m/z=769.137863, RT=5.70 min.

**Measurement of de novo lipid synthesis by 14C labeling and TLC**

To monitor *de novo* mycolic acid biosynthesis, *Mtb* growing in Sauton’s liquid medium was adjusted to OD₆₀₀ 0.5, treated with the indicated concentrations of C10 and/or INH in 1mL final volume, and immediately exposed to 2μCi/mL ¹⁴C-acetate (Perkin Elmer). After incubation at 37°C for 20 hours, cells were pelleted, resuspended in 2:1 chloroform:methanol, vortexed, then samples were pelleted to remove insoluble cell debris, and the supernatant was transferred to a fresh vial. For TLC, 40μL of the sample was added dropwise to a HPTLC plate coated with silica gel 60 matrix (Sigma). TLC plates were developed in 75:10:1 chloroform:methanol:H₂O and imaged by phosphorimaging on a Typhoon laser-scanner (Cytiva). Band intensity was quantified in ImageJ, normalized to the total intensity in the whole lane, and the fold change was quantified relative to the untreated control. To assign putative identities to bands, standards for ¹⁴C-acetate, TDM, free mycolic acid, and oleic acid were included. TDM purified from H37Ra (Invivogen) was derivatized to generate a free mycolic acid standard using methods similar to those previously described. Briefly, 100μL of 0.5mg/mL TDM in isopropanol was subjected to an alkaline ester hydrolysis by mixing with 2μL H₂O and 5μL of 10M KOH, and heating to 90°C for 1 hour to ensure efficient saponification. The resulting mycolic acids were purified from the trehalose by
neutralizing the reaction with 50 μL 1.2M HCl, adding 100 μL chloroform, 100 μL H2O, vortexing, and separating the organic phase from the aqueous layer to obtain free mycolic acids in chloroform.

3.7 Acknowledgements

We thank Drs. Michael Glickman and Allison Fay for helpful guidance with the $^{14}$C labeling experiments. We thank GTAC in the Department of Genetics at WUSTL School of Medicine for help with genomic analysis. The Center is partially supported by NCI Cancer Center Support Grant #P30 CA91842 to the Siteman Cancer Center and by ICTS/CTSA Grant# UL1TR002345 from the NCRR and NIH Roadmap for Medical Research. This publication is solely the responsibility of the authors and does not necessarily represent the official view of NCRR or NIH. This work was supported by a National Science Foundation Graduate Research Fellowship DGE-1745038 (G.A.H.), NIH T32AI007172 (E.W.), a Beckman Young Investigator Award from the Arnold and Mabel Beckman Foundation (C.L.S.), an Interdisciplinary Research Initiative grant from the Children's Discovery Institute of Washington University and St. Louis Children's Hospital (C.L.S.), NIH R01 AI134847 (C.L.S. and F.A), and NIH R35 ES028365 (G.P.J.). Parts of this project were also supported by the Swedish Research Council 2018-04589 and 2021-05040J (F.A.), the Kempe Foundation SMK-1755 (F.A.), the Erling-Persson Foundation (F.A.), and support under the framework of the JPIAMR – Joint Programming Initiative on Anti-microbial Resistance 2018-00969 (F.A.).

Author Contributions: G.A.H. and C.L.S. designed the experiments and wrote the manuscript. G.A.H. and E.R.W. performed the experiments with *Mtb*. K.C. performed and analyzed the mass spectrometry experiments with guidance from G.J.P.. S.S. synthesized C10 with guidance from F.A.. All authors contributed to interpreting the data and editing the manuscript.
Competing Interests: The authors have no competing financial interests to declare, but acknowledge that C.L.S. and F.A. have ownership in the company QureTech Bio AB that licenses C10 and, therefore, may financially benefit if the company is successful in marketing its product.
3.8 Figures:

Figure 3.1: Isolation and characterization of C10-resistant mutants. (A) Chemical structure of C10. (B) WT *Mtb* was cultured in Sauton’s medium containing the indicated concentration of C10, and growth was measured by OD$_{600}$ over time. (C) WT *Mtb* was spread on Sauton’s agar medium containing the indicated concentration of C10 and incubated at 37°C for 3 weeks. (D) Whole genome sequencing of 11 C10-resistant mutants revealed 3 groups of mutants. The mutant loci are depicted along with the resultant nucleotide or amino acid change, and the GHTB strain numbers indicate the mutant isolates that harbored the depicted mutation. Representative strains GHTB136, GHTB146, and GHTB149 were selected for follow up studies. (E) The indicated strain of *Mtb* was cultured in the presence of increasing concentrations of C10 for 1 week, and the % inhibition of Mtb growth and metabolism was determined using the resazurin assay, n=6. (F) The indicated strain of *Mtb* was cultured in Sauton’s liquid medium containing 0 or 25μM C10 for 24 hours before ATP levels were measured by the BacTiter Glo assay. The relative luminescence units (RLU) were normalized to the optical density (OD$_{600}$) of the culture to control for differences in cell density. Fold change in ATP levels were calculated relative to the 0μM C10 control for each strain, n=4-7. A 2-way ANOVA with Tukey’s post test was performed to determine statistically significant differences across samples. Select comparisons are depicted in the figure. ns not significant and **** P<0.0001.
Figure 3.2: Depletion of ATP by C10 is neither necessary nor sufficient to potentiate the bactericidal activity of INH. (A) WT, (B) GHTB136, or (C) GHTB146 Mtb was cultured in Sauton’s liquid medium containing the indicated concentrations of C10 and INH and the CFU/mL were enumerated, n=5. (D) WT Mtb was cultured in Sauton’s liquid medium containing 25μM C10 or 0.05μg/mL BDQ for 24 hours before ATP levels were measured by the BacTiter Glo assay. The RLU were normalized to the OD$_{600}$ of the culture to control for differences in cell density. Fold change in ATP levels were calculated relative to the DMSO control, n=5-7. (E) WT Mtb was cultured in Sauton’s liquid medium containing the indicated concentrations of C10, BDQ, and/or INH and the CFU/mL were enumerated, n=5-7. A 2-way ANOVA with Tukey’s post test was performed to determine statistically significant differences across samples. Select comparison are depicted in the figure. ns not significant, ** P<0.01, and **** P<0.0001.
Figure 3.3: Forward genetic selection results in isolation of a $katG^{W198*}$ mutant that is resistant to INH even in the presence of C10. (A) The indicated strain of Mtb was spread on Sauton’s agar medium containing 25μM C10 and/or 0.5μg/mL INH and incubated at 37°C for 3 weeks. (B) WT (C) $katG^{F56}$, (D) $katG^{W238L}$, and (E) $katG^{W198*}$ Mtb was cultured in Sauton’s liquid medium containing the indicated concentrations of C10 and INH and the CFU/mL were
enumerated. (F) *Mtb* harboring the indicated *katG* allele was cultured in Sauton’s liquid medium containing 0, 10, or 25μM C10 for 24 hours before ATP levels were measured by the BacTiter Glo assay. The RLU were normalized to the OD$_{600}$ of the culture to control for differences in cell density. Fold change in ATP levels were calculated relative to the 0μM C10 control for each strain, n=3-11. (G) The indicated strain of *Mtb* was cultured in the presence of increasing concentrations of C10 for 1 week, and the % inhibition of *Mtb* growth and metabolism was determined using the resazurin assay, n=3. A 2-way ANOVA with Tukey’s post test was performed to determine statistically significant differences across samples. Select comparison are depicted in the figure. ns not significant, * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001.
Figure 3.4: Some level of KatG expression and activity is necessary for C10 to potentiate INH. (A) Western blot against whole cell lysate from the indicated strain of *Mtb*. CarD was used as a loading control in this experiment. (B) Diagram of KatG protein indicating the required catalytic residues in the protein as well as the variant proteins expressed in each *katG* mutant. (C-G) The indicated strain of *Mtb* was treated with or without 0.25μg/mL INH and growth was monitored over time by the optical density (OD$_{600}$), n=4. (H) ΔkatG *Mtb* was spread on Sauton agar medium containing 25μM C10 and/or 0.5μg/mL INH and incubated at 37°C for 3 weeks before images were taken. (I) ΔkatG *Mtb* was cultured in Sauton’s liquid medium containing 0, 10, or 25μM C10 with or without 0.25μg/mL INH and the CFU/mL were enumerated on 7H11 agar with no antibiotics to determine the number of viable bacteria in each culture at days 0 and 10 of treatment. A 2-way ANOVA with Tukey’s post test was performed to determine statistically significant differences. ns not significant, **** P<0.0001.
Figure 3.5: **C10 enhances the bacterial sensitivity to INH-NAD without changing its activation.** (A-D) Purified KatG protein (200nM) was incubated in 50mM potassium phosphate buffer pH 7.0 containing INH, NAD⁺, and 50μM MnCl₂ with 0, 10, or 25μM C10, and samples were taken at the indicated time points and analyzed via LC/MS. For each ion, the peak intensity was calculated as the area under the curve for (A) INH-NAD, (B) INH, (C) NAD⁺, and (D) C10, n=3. (E) Either WT (top) or katG₆₆ Mtb (bottom) was cultured in Sauton’s liquid medium in the presence of 0, 5, or 25μM C10 for 3 days, and whole cell lysate was collected for a Western blot using a monoclonal α-KatG antibody or an α-RpoB antibody as a loading control, n=3. Note that the KatG blot from the katG₆₆ samples has a high amount of background since the exposure time for this blot is substantially longer than for WT samples due to the very low level of KatG protein in this strain. (F) WT or katG₆₆ Mtb was cultured in Sauton’s liquid medium in the presence of 0, 10, or 25μM C10 for 6 days, and whole cell lysate was collected to measure the catalase activity of the sample as a proxy for KatG activity, n=2. Catalase activity is normalized to the amount of total protein in the whole cell lysate sample. Note that lysate from the ΔkatG mutant, n=3, harbors no detectable amount of catalase activity compared to buffer alone, n=3, demonstrating that the assay is specific for KatG. (G) Mtb with the indicated katG allele was cultured for 3 days in Sauton’s liquid medium in the presence and absence of 25μM C10 and/or 0.25μg/mL INH, after which polar metabolites were extracted from the culture and analyzed by LC/MS. The area under the curve was calculated by the integration of the peak to determine the peak intensity of INH-NAD in each bacterial sample, n=3. Statistically significant differences were identified by a 2-way ANOVA with Tukey’s post test, and the relevant comparisons are indicated on the graph. ns not significant, *** P<0.001, **** P<0.0001.
Figure 3.6: C10 enhances the vulnerability of Mtb to InhA inhibition without altering bacterial InhA activity. (A) WT Mtb was cultured in Sauton’s liquid medium with the indicated concentrations of C10, INH, and/or NITD-916, and CFU/mL were enumerated on day 0 and 10 of treatment to determine the number of viable bacteria in each sample, n=6. (B) WT or katG\textsuperscript{W328L} Mtb was cultured in Sauton liquid medium, treated with 25μM C10 and/or 0.25μg/mL INH, and immediately exposed to 2μCi/mL of 14\textsuperscript{C}-labeled acetate. After 20 hours, lipids were extracted and analyzed by TLC to measure the de novo synthesis of mycolic acids and other lipids. The TLC plate was developed with 75:10:1 Chloroform:Methanol:H\textsubscript{2}O and radioactivity was analyzed by phosphorimaging. Bands corresponding to free mycolic acid (MA), fatty acid, trehalose dimycolate (TDM), and 14\textsuperscript{C}-acetate were identified by comigration with a standard. Trehalose monomycolate (TMM) is indicated with a * to emphasize that this lipid is putatively identified, and not correlated with a standard. The plate in (B) is representative of 3 biological replicates, and the standards and additional replicates are shown in Supplementary Figure S6. (C-D) The intensity of bands corresponding to (C) TDM or (D) free MA were quantified in Image J, and normalized to the DMSO sample, with each WT sample being normalized to WT DMSO and each katG\textsuperscript{W328L} sample being normalized to katG\textsuperscript{W328L} DMSO in order to compare across replicates on separate plates, n=3. Statistically significant differences were determined by 2-way ANOVA and select pairwise comparisons are depicted in the figure. ns not significant, *** P<0.001, **** P<0.0001.
Figure 3.7: C10 antagonizes the arabinogalactan inhibitors EMB and BTZ043 while potentiating the antibiotics meropenem and delamanid. WT Mtb was cultured in Sauton’s liquid medium with the indicated concentration of C10 in the presence and absence of 0.25μg/mL INH, 2μg/mL EMB, 0.25μg/mL BTZ043, 2.5μg/mL meropenem and clavulanate (Mero/Clav), 0.25μg/mL delamanid, or 0.25μg/mL RIF and the CFU/mL were enumerated on days 0 and 10 of treatment to determine the number of viable bacteria in each sample, n=3-6. EMB and BTZ043 both inhibit arabinogalactan biosynthesis, whereas Mero/Clav targets peptidoglycan biosynthesis, and delamanid inhibits both Mtb respiration and the biosynthesis of some mycolic acids. RIF inhibits the Mtb RNA polymerase, and serves as a control compound that does not directly impact the Mtb cell wall. Statistically significant differences were determined by performing a two-way ANOVA with Tukey’s post-test, and relevant comparisons are depicted on the graph. ns not significant, * P<0.05, ** P<0.01, **** P<0.0001.
3.9 Supplementary figures

Supplementary Figure 3.S1: The mutations in GHTB136 and GHTB146 impact the expression of neighboring genes. (A-B) The expression of the indicated genes was monitored in (A) GHTB136 or (B) GHTB146 compared to WT *Mtb* by qRT-PCR, n=3. A map of the gene locus as well as the mutation present in the respective mutant strain is depicted above each graph. A 2-way ANOVA with Tukey’s post test was performed to identify statistically significant differences between samples, ****P<0.0001, ***P<0.001, ns not significant.
Supplementary Figure 3.S2: Basal ATP levels in C10-resistant mutants. The indicated strain of *Mtb* was cultured in Sauton’s liquid medium and ATP levels were measured by the BacTiter Glo assay. Data was collected from either 2 (GHTB149) or 3 (WT, GHTB136, GHTB146) independent experiments performed on separate days, n=4-7. The relative luminescence units (RLU) were normalized to the OD$_{600}$ of the culture to control for differences in cell density. A one-way ANOVA was performed to determine if any of the strains had significantly different levels of ATP at baseline. ns, not significant.
Supplementary Figure 3.S3: C10-resistant mutants are not cross-resistant to direct ETC inhibitors. (A-C) The indicated strain of *Mtb* was cultured in the presence of increasing concentrations of (A) BDQ or (B) CCCP for 1 week, and the % inhibition of *Mtb* growth and metabolism was determined using the resazurin assay, n=3. A best fit curve was determined in GraphPad Prism.
Supplementary Figure 3.S4: C10 does not increase KatG catalase activity or thermal stability. (A) Purified *Mtb* KatG protein (25nM) was incubated with or without 25μM C10 and/or 0.25μg/mL INH in 50mM potassium phosphate buffer pH 7.0, and the catalase activity, or H$_2$O$_2$ degradation, was monitored through a decrease in absorbance over time at λ$_{abs}$=240nm in a UV/Vis spectrophotometer, n=3. The data in the graph was fitted with the Michaelis-Menten equation in GraphPad Prism to determine the V$_{max}$ and K$_M$ of KatG in each condition, which are indicated in the figure legend. (B-C) Purified *Mtb* KatG protein (1.1μM) was incubated with (B) INH or (C) C10 in 50mM potassium phosphate buffer pH 7.0 with 50μM MnCl$_2$ before mixing samples with Sypro Orange, incubating at increasing temperatures, and monitoring fluorescence over time. The melting temperature (T$_m$) was calculated by fitting curves with a Boltzmann sigmoidal equation in GraphPad Prism, and the ΔT$_m$ was calculated as the difference between the T$_m$ of each sample and the average of the untreated control samples (n=5). Statistically significant differences were identified by performing a 1-way ANOVA with Tukey’s post test, and data points that are statistically significantly different from the untreated control are indicated with stars. * P<0.05, ** P<0.01.
Supplementary Figure 3.S5: C10 does not potentiate killing by ETH. (A) WT Mtb was cultured in Sauton’s medium containing the indicated concentration of C10, INH, and/or ETH and the CFU/mL were enumerated on day 0 and 10 of treatment to determine the number of viable bacteria in each sample, n=5. Statistically significant differences were identified by performing a two-way ANOVA with Tukey’s post-test, and relevant comparisons are depicted in the graph. ns, not significant, * P<0.05, ** P<0.01, **** P<0.0001. (B) The relative expression of ethA, mymA, and ethA2 was determined after exposing WT Mtb to 25μM C10 for 48 hours and performing RNA-seqencing. The data in panel B is reproduced from our previously published RNA-seqencing dataset. The data is graphed as the Log2(Fold Change) in the RNA level in C10-treated samples relative to the DMSO treated control, and the p value of the difference between C10-treated and control samples is indicated on the graph, calculated as the adjusted p value from the DESeq2 analysis pipeline.
Supplementary Figure 3.S6: C10 does not alter mycolic acid biosynthesis in the presence or absence of INH. (A) WT or katG\(^{W328L}\) \(Mtb\) was cultured in Sauton liquid medium, treated with 25\(\mu\)M C10 and/or 0.25\(\mu\)g/mL INH, and immediately exposed to 2\(\mu\)Ci/mL of \(^{14}\)C-labeled acetate. After 20 hours, lipids were extracted and analyzed by TLC to measure the \textit{de novo} synthesis of mycolic acids and other lipids. The TLC plate was developed with 75:10:1 Chloroform:Methanol:H\(_2\)O and radioactivity was analyzed by phosphorimaging. Bands corresponding to free mycolic acid (MA), fatty acid, trehalose dimycolate (TDM), and \(^{14}\)C-acetate were identified by comigration with a standard. Cold standards were imaged by coating the TLC plate in phosphomolybdic acid and charring. Trehalose monomycolate (TMM) is indicated with an * to emphasize that this lipid is putatively identified, and not correlated with a standard. (B-C) The intensity of bands corresponding to (B) TMM and (C) fatty acids were quantified in Image J, and normalized to the DMSO sample, with each WT sample being normalized to WT DMSO and each katG\(^{W328L}\) being normalized to katG\(^{W328L}\) DMSO in order to compare across replicates, \(n=3\). Note that one of the images in panel A, is the same image that is presented in Figure 6 in the main text, reproduced here for comparison to the other replicates. Statistically significant differences were determined by 2-way ANOVA and select pairwise comparisons are depicted in the figure. ns not significant, *** \(P<0.001\), **** \(P<0.0001\).
Supplementary Figure 3.S7: Delamanid depletes bacterial ATP levels. WT Mtb was cultured in Sauton’s medium containing 0 or 25μM C10 in the presence and absence of 0.25μg/mL INH and 0.25μg/mL delamanid for 24 hours before ATP levels were measured by the BacTiter Glo assay. The RLU were normalized to the OD_{600} of the culture to control for differences in cell density, n=3. Statistically significant differences were determined by performing a two-way ANOVA with Tukey’s post-test, and relevant comparisons are depicted on the graph. ns not significant, ** P<0.01, **** P<0.0001.
### 3.10 Tables

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Chapter 4:  C10 impairs energy metabolism in 
*Mycobacterium tuberculosis*

This chapter is un-published work that may comprise part of a future manuscript that is submitted for publication with the following co-contributors:

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Preface:

The work in this chapter is not published, and it is my hope that someday a member of the Stallings lab will find this data helpful in their own studies and will build on these preliminary findings to craft a complete and cohesive story. GAH performed the experiments in this chapter under the supervision of CLS, with assistance from co-authors. KC made this work possible by performing extensive liquid chromatography-mass spectrometry under the supervision of GJP using *Mycobacterium tuberculosis* extracts generated by GAH. ERW has recapitulated some of the metabolite supplementation data in independent assays, and our work fortuitously converged on similar findings. SS synthesized the C10, PD217, and SS451 used in these experiments under the supervision of FA.
4.1 Abstract:

*Mycobacterium tuberculosis* (*Mtb*) drug resistance represents a critical threat to global health. Discovery of small molecules that inhibit *Mtb* through novel mechanisms of action is essential to develop new therapeutics to target these resistant strains. *Mtb* energy homeostasis is a validated drug target, with a number of anti-tuberculosis drugs currently in clinical development that target the mycobacterial electron transport chain and oxidative phosphorylation. Our lab previously discovered the compound C10, which inhibits *Mtb* respiration through an unknown mechanism. Remarkably, in addition to inhibiting *Mtb* growth at high concentrations, at sub-inhibitory concentrations C10 potentiates the bactericidal activity of INH, even against a subset of otherwise INH-resistant mutants. Understanding how C10 elicits these effects could reveal new therapeutic targets to inhibit *Mtb* growth and enhance the activity of INH. In the current study, we further characterize a C10-resistant isolate, demonstrating that it has basal metabolic changes resulting in increased glycolytic intermediates. Through metabolic profiling and chemical rescue screens, we determine that the toxicity of C10 is likely mediated through depletion of essential *Mtb* metabolites, especially pyruvate. These studies highlight that by understanding how C10 perturbs *Mtb* central carbon metabolism, we might uncover new mechanisms to target this dangerous pathogen and ultimately circumvent antibiotic resistance.

4.2 Introduction:

In the face of the antibiotic resistance crisis, there is a dire need to identify novel therapeutics that can circumvent the antibiotic resistance mechanisms in resistant strains. In the global tuberculosis pandemic, INH-resistance is the most common form of mono-resistance. Given the remarkable ability of C10 to enhance the bactericidal activity of INH and restore
antibiotic susceptibility in a subset of otherwise INH-resistant mutants, we are interested in understanding the precise mechanism of action of C10. We previously performed transcriptomic profiling of C10-treated Mtb and found that C10 induced a transcriptional signature consistent with disruptions in Mtb energy metabolism (Chapter 2). In follow-up assays, we demonstrated that C10 inhibits Mtb respiration and causes depletion of bacterial ATP levels, confirming that C10 disrupts Mtb energy homeostasis. Furthermore, while at the concentrations that potentiate INH (5-25μM), C10 has poor growth-inhibitor activity, we found that increasing the concentration of C10 caused a dose-dependent decrease in bacterial growth.

Taking advantage of the toxicity caused by this high concentration of C10, I performed a forward genetic selection to isolate C10-resistant mutants (Chapter 3). I found that 2 mutants that exhibit high levels of C10 resistance were insensitive to the ATP-depleting effects of C10, maintaining ATP levels even during C10 treatment. Using these resistant mutants, I found that depletion of ATP by C10 was not required for its INH-potentiating effects. Furthermore, depletion of ATP using the ATP synthase inhibitor BDQ was not sufficient to potentiate the bactericidal activity of INH. Together, these findings indicate that C10 must impart another effect on the bacteria that is not reversed in the resistant mutants that leads to INH potentiation.

However, its inhibitory effects on Mtb growth suggest that C10 could be developed on its own as a mycobacterial growth inhibitor. Its dual activity, inhibiting Mtb growth on its own at high concentrations and enhancing the bactericidal activity of INH at sub-inhibitory concentrations, indicates that C10 or a related compound could be a useful addition to the current frontline antibiotic regimen.

Mtb energy metabolism is a validated drug target. In 2012, the United States Food and Drug Administration approved Bedaquiline (BDQ), which was the first new anti-tuberculosis
antibiotic approved in over 40 years. BDQ targets the mycobacterial ATP synthase, inhibiting oxidative phosphorylation. Inspired by the potent anti-\textit{Mtb} activity of BDQ, multiple groups have identified antimycobacterial compounds that target \textit{Mtb} bioenergetics, some of which are currently in clinical development. Since C10 on its own can inhibit \textit{Mtb} growth by disrupting energy homeostasis, it is possible that the antimycobacterial effect of C10 could be leveraged to develop a novel inhibitor of \textit{Mtb} that could prove useful in the clinic even in the absence of INH. To this end, our lab has undertaken extensive structure activity relationship studies to identify C10 analogs with more potent activity against \textit{Mtb} (Anne Mayer Bridwell & Souvik Sarkar, et al. unpublished). Through this work, we have identified two C10 analogs, PD217 and SS451, that each have a half-maximal inhibitory concentration (IC$_{50}$) in the sub-micromolar range.

However, a better understanding of the mechanism by which C10 disrupts \textit{Mtb} energy metabolism would improve our ability to optimize this class of small molecules to inhibit \textit{Mtb}. In the current study, I further characterize the mechanism of C10-resistance in the mutants that maintain ATP levels during treatment with C10 to understand how these mutants overcome the effects of C10 on energy homeostasis. By contrasting the physiological response of \textit{Mtb} to C10 to that of BDQ, our work reveals that C10 inhibits \textit{Mtb} energy metabolism through a distinct mechanism, not consistent with inhibition of oxidative phosphorylation. Furthermore, metabolic profiling and chemical rescue experiments highlight the potential impact of C10 on central carbon metabolism. While the precise mechanism of action of C10 remains unknown, our studies reveal that C10 inhibits \textit{Mtb} growth by perturbing central carbon metabolism. Therefore, C10 and related compounds represent a class of \textit{Mtb} growth inhibitors that compromise energy homeostasis by limiting bacterial metabolism.

\section*{4.3 Results and Discussion:}
4.3.1 The \textit{lpdA-glpD2} over-expressing strain GHTB146 is cross-resistant to C10 analogs

In a collaboration between the Stallings lab and the Almqvist lab at Umeå University in Sweden, we have performed structure activity relationship studies to identify C10 analogs with more potent activity against \textit{Mtb} (Anne Mayer Bridwell & Souvik Sarkar, et al. unpublished). Through this work, we identified the more potent C10 analogs PD217 and SS451, which share the bicyclic 2-pyridone backbone of C10 but have slightly altered substituents on the ring backbone (Figure 4.1A). To directly compare the activity of C10, PD217, and SS451, I used the resazurin microplate assay to quantify the half-maximal inhibitory concentration (IC$_{50}$) of each of these compounds. This assay takes advantage of the redox-sensitive dye resazurin, which is blue in its oxidized form but becomes reduced to the fluorescent pink product resorufin as a result of bacterial growth and metabolism. Therefore, fluorescence can be monitored as a proxy for bacterial growth and metabolism. Compared to C10, which has an IC$_{50}$ of 21μM in this experiment, the IC$_{50}$s of PD217 and SS451 were 0.31μM and 0.20μM, respectively (Figure 4.1B). Therefore, PD217 is 67-fold and SS451 is 105-fold more potent than C10 in this assay (Figure 4.1B).

In work described in Chapter 3, we identified 3 mutants with resistance to C10. GHTB136 harbors an intergenic mutation between the putative S-adenosylmethionine (SAM)-methyl transferase \textit{Rv0731c} and the secY-\textit{adk-mapA} operon, leading to >100-fold up-regulation of the \textit{Rv0731c} gene and 2-6-fold up-regulation of the \textit{secY-\textit{adk-mapA}} operon compared to WT (Chapter 3). GHTB146 harbors an intergenic mutation between the \textit{lpdA-glpD2} operon and the uncharacterized gene \textit{Rv3304}, leading to 4-8-fold up-regulation of the \textit{lpdA-glpD2} operon and no change in expression of \textit{Rv3304} (Chapter 3). The third strain, GHTB149, harbors a missense mutation within the putative SAM-methyl transferase \textit{Rv0830} that results in the substitution of
valine for a leucine residue at position 292 (L292V).

To determine if these mutations confer cross-resistance to the more potent C10 analogs PD217 and SS451, I quantified the sensitivity of GHTB136, GHTB146, and GHTB149 to these analogs using a resazurin microplate assay. I found that GHTB136 and GHTB146 were respectively 13- and 8-fold more resistant to PD217, whereas the IC$_{50}$ of PD217 against GHTB149 was not significantly different from WT (Figure 4.1C). Therefore, the mutations in GHTB136 and GHTB146 mediate resistance to PD217 in addition to C10. Additionally, while both GHTB136 and GHTB146 had significant increases in the IC$_{50}$ of SS451 compared to WT, GHTB136 only exhibited a modest 2-fold increase in the IC$_{50}$ whereas GHTB146 exhibited a 13-fold increase in the IC$_{50}$ of SS451 compared to WT (Figure 4.1D). Together these findings indicate that while both GHTB136 and GHTB146 exhibit significant shifts in the IC$_{50}$ of PD217 and SS451 compared to WT, only GHTB146 exhibits high level resistance to both of these C10 analogs. The mechanism of resistance in GHTB146 is therefore sufficient to confer cross-resistance to PD217 and SS451 in addition to C10. By understanding how GHTB146 is resistant to these related compounds we might gain insight into their mechanism of action.

4.3.2 GHTB146 exhibits increased flux through the glycerol assimilation pathway

The GHTB146 strain harbors a mutation in the promoter region of the $lpdA$-$glpD2$ operon that causes significant up-regulation of this operon (Chapter 3). Since this strain is cross-resistant to all the C10 analogs we tested, we sought to understand how up-regulated expression of $lpdA$ or $glpD2$ might confer resistance to this class of compounds. LpdA is proposed to be a dehydrogenase that can oxidize or reduce NAD(H) or NADP(H) with concomitant oxidation or reduction of menaquinone/menaquinol in the plasma membrane. Biochemical studies suggest that LpdA has a
preference for NADP(H) over NAD(H)\textsuperscript{253}. GlpD2 has not been characterized in \textit{Mtb}, but based on homology to the GlpD enzyme of \textit{E. coli}\textsuperscript{254}, it is predicted to be a glycerol-3-phosphate dehydrogenase. This enzyme can interconvert glycerol-3-phosphate and dihydroxyacetone phosphate (DHAP) with concomitant oxidation or reduction of menaquinone/menaquinol in the membrane. Therefore, GlpD2 likely functions in glycerol metabolism, perhaps in association with LpdA.

Glycerol-3-phosphate is an intermediate in the glycerol assimilation pathway. In this pathway GlpK first phosphorylates glycerol to glycerol-3-phosphate, which is then oxidized to DHAP via the activity of GlpD2 or one of 3 other glycerol-3-phosphate dehydrogenase enzymes in \textit{Mtb}, GpsA, GpsA2, or GlpD1\textsuperscript{255}. DHAP is an intermediate in the glycolysis/gluconeogenesis pathway in \textit{Mtb}, and therefore assimilation of glycerol through this pathway can feed into these downstream pathways. Notably, using chemical or genetic inactivation of GlpK it was shown that the glycerol assimilation pathway is required for growth \textit{in vitro} in media containing glycerol as the primary carbon source, but is dispensable in the presence of other carbon sources such as glucose\textsuperscript{256,257}. Furthermore, GlpK was dispensable for \textit{Mtb} growth and survival in mice, indicating that free glycerol may not be an essential carbon source in the host\textsuperscript{256}. However, glycerol-3-phosphate can be generated through multiple pathways, including the synthesis or degradation of di- and triacylglycerol lipids\textsuperscript{176}. Since the \textit{Mtb} genome encodes 4 different enzymes that can interconvert glycerol-3-phosphate and DHAP, this particular step in metabolism could be important for \textit{Mtb} survival in the host, even if the phosphorylation of free glycerol by GlpK is dispensable, although this has not been tested directly. The directionality of the glycerol-3-phosphate dehydrogenases cannot be predicted simply based on the primary amino acid sequence, so these 4 enzymes could potentially function reversibly to generate DHAP from glycerol-3-
phosphate or to synthesize glycerol-3-phosphate from DHAP in the opposite direction.

Our culture media contains 6% glycerol as the primary carbon source for \textit{Mtb} growth, suggesting that glycerol assimilation is likely occurring in our \textit{in vitro} culture conditions. Based on the predicted function of GlpD2, we hypothesized that the GHTB146 mutant has altered flux through this pathway. It is possible that up-regulation of GlpD2 could lead to increased conversion of glycerol-3-phosphate to DHAP, or to increased synthesis of glycerol-3-phosphate from DHAP in the opposite direction. To distinguish between these possibilities, we collected polar metabolites from WT and GHTB146 \textit{Mtb} and used liquid chromatography-mass spectrometry (LC-MS) to quantify the basal levels of glycerol-3-phosphate and DHAP in these \textit{Mtb} strains. We found that the GHTB146 strain exhibited a significant >2-fold decrease in the abundance of glycerol-3-phosphate and a significant >2-fold increase in the abundance of DHAP compared to WT, indicating that up-regulation of \textit{lpdA-glpD2} likely leads to increased flux through this pathway towards DHAP, rather than in the opposite direction (Figure 4.2). Furthermore, when we examined the metabolites that are downstream from DHAP in glycolysis and gluconeogenesis, we found that GHTB146 harbors significantly increased basal levels of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6BP), and pyruvate (Figure 4.2). This metabolic profile suggests that the increased carbon assimilated through the activity of GlpD2 feeds into downstream metabolic pathways, leading to elevated pools of hexose phosphates and the glycolytic product pyruvate.

In our previous work, we showed that the other mutant with high-level C10 resistance, GHTB136, exhibited increased expression of the putative SAM-methyltransferase \textit{Rv0731c} (Chapter 3). Since the substrate of \textit{Rv0731c} is unknown, we wondered whether this enzyme might also be involved in central carbon metabolism, either directly by methylating a central carbon
metabolite or indirectly by modifying a central carbon metabolism enzyme. We collected polar metabolites from GHTB136 and performed a similar basal metabolite analysis to measure the abundance of various metabolites within the bacteria. GHTB136 did not have a similar metabolic profile to GHTB146, supporting that the mechanism of resistance in these two strains is likely distinct. Notably, of the 60 metabolites that we measured in the GHTB136 strain, only 3 were significantly different from WT (Supplementary Table 4.S1). There was a significant >2-fold increase in S-adenosyl homocysteine (SAH) levels compared to WT, consistent with increased SAM-methyltransferase activity, which generates the byproduct SAH. Additionally, GHTB136 exhibited modest <2-fold increase in the amino acid lysine, and a significant >2-fold increase in the amino acid alanine (Supplementary Table 4.S1).

Curiously, the GHTB146 strain also exhibited significantly >2-fold increased alanine levels (Supplementary Table 4.S1). Alanine can be interconverted with pyruvate through the reversible activity of \textit{Mtb} alanine dehydrogenase, indicating that GHTB146 could harbor increased alanine pools as a downstream result of the excess pyruvate in this strain. In contrast, since GHTB136 exhibited increase alanine, but no difference in pyruvate levels compared to WT, this mutant could be accumulating alanine through a different mechanism. While this analysis did not reveal a clear substrate for \textit{Rv0731c}, the shared perturbation in alanine levels in both GHTB136 and GHTB146 highlights that altered pyruvate alanine homeostasis could be linked to the mechanism of resistance to C10.

4.3.3 C10 limits pyruvate metabolism

To investigate how C10 impacts \textit{Mtb} metabolism, I performed metabolomic profiling on \textit{Mtb} treated with 25\(\mu\)M C10 for 4 days. I included the GHTB136 and GHTB146 mutants in addition to WT \textit{Mtb} in order to identify C10-induced metabolic changes that are unique to WT and
are reversed in the C10-resistant mutants. After 4 days of treatment, C10 caused several statistically significant alterations in central carbon metabolism. Notably, glycerol-3-phosphate is significantly 1.2-fold increased and DHAP is 1.6-fold decreased in WT *Mtb* exposed to C10 (Figure 4.3), indicating that C10 has a modest but statistically significant impact on the levels of metabolites in the glycerol assimilation pathway. Notably, neither glycerol-3-phosphate nor DHAP are significantly altered by C10 in GHTB136 or GHTB146 (Figure 4.3).

In addition to the effects on the glycerol assimilation pathway, metabolic changes induced by C10 include (1) a general decrease in sugars, including the acetylated amino-sugars N-acetyl-glucosamine (GlcNAc)-phosphate, UDP-GlcNAc, and UDP-N-acetyl-muramic acid (UDP-MurNAc); (2) decreased glycolytic intermediates F1,6BP, phosphoenolpyruvate (PEP), and pyruvate; (3) altered TCA cycle homeostasis, including increases in the oxaloacetate proxy aspartate, citrate, and aconitate, with a concomitant decrease in α-ketoglutarate; and (4) altered branched chain amino acid metabolism, including decreased isoleucine and leucine, with an accompanying increase in the methylcitrate cycle (MCC) intermediate 2-methylcitrate (Figure 4.3). While all of these changes are statistically significant, the most striking change is the 7.7-fold depletion of pyruvate after 4 days of C10 treatment, which is slightly dampened in the GHTB136 strain and completely reversed in the *lpdA-glpD2*-overexpressing GHTB146 mutant (Figure 4.3). This data supports that C10 treatment decreases bacterial pools of pyruvate, which are restored in the GHTB146 strain, likely due to the increased assimilation of glycerol.

It should be noted that by measuring the bulk changes in metabolite pools it is possible to overlook relevant metabolic changes due to the myriad of compensatory pathways in *Mtb*. Furthermore, it is impossible to determine if elevated pools of a particular metabolite are a result of increased synthesis or decreased utilization of that metabolite, and conversely whether depleted
pools are a result of increased utilization or decreased synthesis. In order to precisely identify specific metabolic steps that are impacted, it is necessary to perform metabolic flux analysis, using a non-radioactive heavy $^{13}$C-labeled carbon source to track which pathways are increased and which are decreased during C10 treatment. This kind of analysis would enable us to definitively identify which step(s) of central carbon metabolism are specifically impacted by C10 treatment. Still, the bulk changes in central carbon metabolites in response to C10 demonstrate clearly that C10 alters *Mtb* metabolism, ultimately resulting in a 7.7-fold decrease in bacterial pyruvate.

4.3.4 The toxicity of C10 is not due to inhibition of the glycerol assimilation pathway

Based on the finding that the *lpdA-glpaD*-overexpressing strain GHTB146 has high-level resistance to C10, PD217, and SS451, in addition to the metabolite data showing that C10 treatment causes modest but significant changes in the abundance of glycerol-3-phosphate and DHAP, we next examined whether C10 inhibited the glycerol assimilation pathway.

While glycerol assimilation is essential for growth in media that contains glycerol as a primary carbon source, this pathway is dispensable in conditions where other carbon sources are provided \(^\text{256,257}\). All of the experiments presented thus far have been performed in Sauton's growth media, which is a chemically defined media that contains 6% glycerol (650mM) as the primary carbon source. Therefore, we reasoned it was possible that C10 depletes pyruvate and other glycolytic intermediates by inhibiting glycerol assimilation, and GHTB146 overcomes this inhibition by increasing the expression of *lpdA-glpaD*.  

To examine if C10 maintained activity in the absence of glycerol, I generated modified Sauton’s media that lacks glycerol and contains 10mM glucose, 10mM pyruvate, 10mM acetate, 1mM propionate, 10mM α-ketoglutarate, or 10mM glutamate as the primary carbon source. First,
to determine if these carbon sources supported *Mtb* growth in the absence of glycerol, I cultured *Mtb* in these media conditions for 1 week, then exposed the bacteria to resazurin and measured the amount of fluorescence as a read out of growth and metabolism (Figure 4.4A). As a control, I included media where no additional carbon source was added. I found that *Mtb* growth and metabolism was supported in media containing glycerol, glucose, pyruvate, or acetate as the primary carbon source (Figure 4.4A). However, *Mtb* cultured in modified Sauton’s media containing propionate, α-ketoglutarate, or glutamate as the primary carbon source did not generate a fluorescent signal that was significantly different from the no carbon source control, suggesting that these carbon sources do not support *Mtb* growth or metabolism in the absence of glycerol (Figure 4.4A).

I then used the resazurin assay to quantify the sensitivity of *Mtb* to C10 in modified Sauton’s medium containing glucose, pyruvate, or acetate as the primary carbon source compared to normal Sauton’s medium containing glycerol. I found that C10 could inhibit *Mtb* in this assay regardless of the primary carbon source used (Figure 4.4B), demonstrating that the growth-inhibitory activity of C10 is not dependent on the glycerol assimilation pathway. Yet, the IC$_{50}$ of C10 was significantly increased during growth on glucose, pyruvate, or acetate compared to the glycerol control, suggesting that the metabolic state can influence the bacterial sensitivity to C10 (Figure 4.4B). Therefore, while we found that C10 slightly perturbs the glycerol assimilation pathway (Figure 4.3), growth on glycerol as the primary carbon source is not necessary for C10 to inhibit *Mtb* (Figure 4.4B), so C10 must inhibit a pathway that is essential even in the absence of glycerol.

**4.3.5 Exogenous supplementation of carbon metabolites rescues C10 toxicity**

Since C10 maintains the ability to inhibit *Mtb* during growth on glycerol, glucose,
pyruvate, and acetate as primary carbon sources, I expect C10 inhibits a pathway that is essential for *Mtb* growth in these conditions. Based on our findings, I hypothesize that C10 disrupts *Mtb* energy homeostasis and inhibits growth by targeting a central carbon metabolism pathway. While we observed a number of metabolic changes in response to C10, it is unclear which metabolic changes contribute to the growth inhibitory activity of C10. We found alterations in sugar metabolism, glycolysis, gluconeogenesis, the TCA cycle, amino acid metabolism, and the MCC. Furthermore, it is possible that C10 targets the biosynthesis of essential co-factors, leading to impacts on multiple metabolic pathways simultaneously. Additionally, our finding that C10 induced 2-methylcitrate, a metabolite in the MCC pathway that is responsible for detoxifying the toxic metabolite propionyl-CoA, suggests that it is possible C10 inhibits growth by inducing propionyl-CoA toxicity.

To investigate if C10 inhibits growth by affecting one or more of these pathways, I performed a targeted chemical supplementation screen to test if exogenous carbon sources or co-factors could rescue the inhibition of *Mtb* by C10 or exacerbate its toxic effects (Figure 4.5). I generated modified Sauton’s media that contained various supplements in addition to the 6% glycerol that is present in the base medium. To investigate if C10 inhibits *Mtb* by restricting sugar metabolism, I included glucose as well as the cell wall sugars arabinose, rhamnose, MurNAc, and GlcNAc. To investigate if C10 compromises glycolysis or gluconeogenesis, in addition to glucose, I included the end-product of glycolysis pyruvate. To investigate if C10 inhibits *Mtb* by interrupting the TCA cycle, I included acetate, which can readily be converted to acetyl-CoA and feed into the TCA cycle, as well as aspartate, which can readily be converted to oxaloacetate, the intermediate that is coupled to acetyl-CoA in the first step of this pathway. I additionally included α-ketoglutarate, one of the metabolites that is significantly depleted during C10 treatment. To
examine if C10 inhibits *Mtb* by altering amino acid metabolism, in addition to aspartate I included the amino acids glutamate, glycine, and serine, as well as the important nitrogen metabolite urea. To investigate if C10 inhibits the synthesis of essential co-factors, I included nicotinamide, a precursor of cofactors such as NAD$^+$ or NADP$^+$, and thiamine, a cofactor required by pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and branched chain α-keto acid dehydrogenase. To determine if C10 inhibits growth through the formation of toxic reactive oxygen species (ROS), I included the ROS scavengers ascorbate and N-acetylcysteine, which should detoxify these ROS. Finally, to investigate if C10 induces propionyl-CoA toxicity, I included exogenous propionate, which should exacerbate the toxic effects of propionyl-CoA accumulation, and vitamin B12, which can alleviate the toxic effects of propionyl-CoA by enabling propionyl-CoA detoxification through an alternative pathway.

I cultured *Mtb* in Sauton’s media containing 6% glycerol with and without these supplements and used the resazurin microplate assay to quantify the IC$_{50}$ of C10 in each of these conditions. Intriguingly, exogenous supplementation with nicotinamide or N-acetylcysteine enhanced the toxicity of C10, causing dose-dependent decreases in the IC$_{50}$ of C10 (Figure 4.5). While it is unclear precisely how these supplements enhance the activity of C10, it is possible that nicotinamide does not promote cofactor biosynthesis in these conditions, and instead causes toxic effects on the bacteria that are exacerbated by C10. Additionally, since N-acetylcysteine is a reducing agent, it is possible that C10 makes *Mtb* more sensitive to reductive stress. Both of these observations warrant further investigation.

However, several carbon source supplements significantly increased the IC$_{50}$ of C10, indicating that they chemically rescue inhibition by C10 (Figure 4.5). I found that exogenous supplementation with glucose, pyruvate, or acetate could rescue inhibition by C10. During
glycolytic metabolism, glucose is oxidized to pyruvate, and then converted into acetyl-CoA through the activity of pyruvate dehydrogenase. Acetyl-CoA then feeds into a myriad of pathways, including the TCA cycle. Therefore, glucose, pyruvate, and acetate metabolism are tightly connected. Our finding that both pyruvate and acetate supplementation can rescue C10 inhibition may indicate that the toxicity of C10 is due to decreased availability of acetyl-CoA. While C10 treatment did not alter the level of acetyl-CoA in WT *Mtb* based on our metabolite profiling experiments (Figure 4.3), these changes could be masked in bulk-metabolite analysis due to the low level of acetyl-CoA maintained in the cell as well as the activity of compensatory pathways. Performing $^{13}$C-flux analysis in future studies could resolve the effect of C10 on this metabolic pathway. Alternatively, acetate supplementation may kinetically inhibit pyruvate dehydrogenase activity, ultimately restoring pyruvate levels in the bacteria.

Furthermore, we found that exogenous supplementation with propionate did not exacerbate the toxicity of C10 (Figure 4.5), demonstrating that C10 does not induce propionyl-CoA toxicity. If anything, exogenously providing 1mM propionate caused an increase in the IC$_{50}$ of C10 (Figure 4.5). The MCC couples propionate with oxaloacetate and ultimately generates succinate and pyruvate as end-products, suggesting that the provision of exogenous propionate may ultimately rescue inhibition by C10 by also feeding into downstream pyruvate metabolism.

Therefore, in addition to our earlier findings that GHTB146 exhibits altered pyruvate homeostasis and that C10 depletes bacterial pyruvate levels, our carbon source supplementation data converges on the idea that pyruvate plays a critical role in the mechanism of C10-mediated growth inhibition. Our data demonstrates that increasing the concentration of pyruvate within the bacteria either through exogenous supplementation (Figure 4.5) or by increased glycerol assimilation (Figure 4.2) mitigates the inhibitory activity of C10, supporting that pyruvate
deprivation contributes to C10-mediated growth inhibition.

Since glucose, pyruvate, acetate, and propionate can all feed into energy generating pathways, we wondered whether the ability of these compounds to rescue C10 inhibition was due to a general increase in energy metabolism in response to these additional carbon sources. To determine if these carbon sources can simply mitigate the effect of decreased ATP production during C10 treatment, I examined whether these supplements could also rescue inhibition of *Mtb* during inhibition of the ATP synthase with BDQ in the resazurin microplate assay (Figure 4.6). I found that while for C10, each of these carbon sources increased the IC$_{50}$ in a dose-dependent manner, consistent with our earlier screening data, the IC$_{50}$ of BDQ remained consistent or was decreased by the supplementation of these exogenous carbon sources (Figure 4.6). These findings clearly demonstrate that the mechanism of action of C10 differs from BDQ, and further support that C10 likely disrupts energy homeostasis by targeting metabolism, not oxidative phosphorylation.

### 4.3.6 Metabolic profiling of Mtb treated with C10 and INH

Our previous data suggested that the depletion of ATP by C10 is neither required nor sufficient to enhance the bactericidal activity of INH (Chapter 3). However, based on our finding that C10 disrupts central carbon metabolism, we wondered if C10 impacts a metabolic pathway upstream of ATP synthesis that separately leads to increased INH sensitivity. In this regard, the depletion of ATP may be a downstream effect of C10 activity, even if it is clearly not the driving cause of the INH potentiation.

To investigate how the metabolic dysregulation caused by C10 treatment might impact INH sensitivity, I performed metabolite profiling on *Mtb* treated with 25μM C10 with and without 0.25μM INH for 3 days. I included BDQ-treated *Mtb*, since BDQ depletes ATP but does not
potentiate killing by INH (Chapter 3), in order to identify metabolic changes that are unique to C10 and might contribute to this enhanced INH sensitivity. Notably, the 3-day time point used in this experiment differs from the 4-day treatment used in Figure 3, making it difficult to directly compare these experiments. Yet, consistent with our previous data (Figure 4.3), we found that C10 caused a decrease in several TCA cycle intermediates, including α-ketoglutarate, and an increase in the oxaloacetate proxy aspartate (Figure 4.7). In contrast to our previous dataset, C10 caused only a modest decrease in pyruvate levels after 3 days of treatment, compared to the striking >7-fold decrease in pyruvate observed after 4 days of treatment, suggesting that the depletion of pyruvate by C10 is likely time dependent.

Compared to C10, BDQ induced a different metabolic profile. Exposure to BDQ caused accumulation of lower glycolytic intermediates (Figure 4.7), consistent with previous reports that inhibition of ATP synthase by BDQ leads to a compensatory increase in glycolytic activity, likely as an attempt to generate ATP by substrate level phosphorylation while oxidative phosphorylation is inhibited. Additionally, BDQ treatment led to increased levels of TCA cycle intermediates, including α-ketoglutarate, causing an opposite effect compared to C10 (Figure 4.7). One of the few metabolic changes that occurred during both C10 and BDQ treatment is a significant increase in pools of the MCC intermediates propionyl-CoA and 2-methylcitrate (Figure 4.7), indicating that activation of the MCC could be a shared response to ATP depletion by both C10 and BDQ.

In contrast, we found that treatment with INH caused a dramatic increase in the accumulation of sugars, including intermediates in the pentose phosphate pathway and upper glycolysis (Figure 4.7). It has been reported since the 1960’s that INH causes an accumulation of sugars in \textit{Mtb} through an unknown mechanism. INH could lead to increased levels of sugars by inhibiting a step in glycolysis or by activating gluconeogenesis, although it remains unclear
how this metabolic adaptation to INH contributes to the bacterial response to this antibiotic. In addition to impacting sugar metabolism, INH caused a decrease in the amount of pyruvate and most TCA cycle intermediates, although the magnitude of this decrease varied across samples (Figure 4.7).

Adding BDQ in combination with INH appeared to reverse a number of the metabolic changes induced by INH and recapitulate the effect of adding BDQ alone, indicating that the metabolic response to BDQ is dominant over that of INH (Figure 4.7). In contrast, adding C10 in combination with INH reversed the effect of INH on glycolytic intermediates, but appeared to exacerbate the decrease in pyruvate and TCA cycle intermediates. Whereas exposing *Mtb* to the combination of C10 and INH caused a precipitous decrease in these metabolites, the combination of BDQ and INH led to an increase in some of these TCA cycle intermediates, highlighting that the metabolic impact of C10 differs from BDQ in the context of INH treatment (Figure 4.7).

The broad depletion of TCA cycle intermediates caused by the combination of C10 and INH is consistent with TCA cycle dysfunction. One of the primary outputs of TCA cycle activity is reduction of NAD$^+$ to NADH, which then donates electrons to the electron transport chain to fuel oxidative phosphorylation. During treatment with INH, *Mtb* activates INH to an INH-NAD adduct that inhibits the lipid synthesis enzyme enoyl-ACP reductase, InhA, by interacting with the enzyme’s NADH binding site. INH-NAD is a competitive inhibitor of InhA, and it has been shown in *Mycobacterium smegmatis* and *Mycobacterium bovis*, that shifting the redox status of NADH/NAD$^+$ to increase the cellular pool of reduced NADH can mediate resistance to INH, likely due to the ability of NADH to out-compete INH-NAD for binding to InhA$^{262,263}$. We therefore wondered whether C10 treatment alone or in combination with INH caused depletion of NADH, which might enhance the ability of INH-NAD to inhibit InhA due to
decreased competition for its active site. To examine this possibility, we quantified the NADH and NAD\(^+\) ratio in *Mtb* extracts treated with C10 and/or INH for 3 or 4 days using LC-MS, and calculated the NADH/NAD\(^+\) ratio to determine if C10 decreases the relative amount of NADH (Figure 4.8). We found that C10 did not significantly change the NADH/NAD\(^+\) ratio compared to the DMSO control. Upon exposure to INH, *Mtb* exhibited an approximate 10-fold increase in the NADH/NAD\(^+\) ratio, consistent with INH-NAD inhibiting the utilization of NADH by InhA, leading to build-up of this reduced co-factor. Still, even in the presence of INH, C10 did not significantly decrease the NADH/NAD\(^+\) ratio compared to INH alone, indicating that the TCA cycle dysfunction observed in this condition is not sufficient to shift the pools of NADH and NAD\(^+\) toward the oxidized state. C10 must have some other effect on the bacteria that is leading to the increased INH sensitivity.

### 4.4 Conclusion:

In this work we have demonstrated that the C10-resistant GHTB146 mutant is cross resistant to PD217 and SS451, suggesting that these newer C10 analogs could be inhibiting *Mtb* through a similar mechanism to C10. Furthermore, we showed that GHTB146 exhibited a basal increase in the glycerol assimilation pathway, leading to accumulation of glycolytic intermediates, including pyruvate. C10 treatment depleted bacterial pyruvate levels, and supplementation with exogenous pyruvate rescued inhibition by C10. Therefore, exposure to C10 is associated with decreased pyruvate pools, and increasing bacterial pyruvate levels promotes resistance to C10, suggesting that the mechanism of growth inhibition is linked to depletion of this metabolite.

Additionally, our findings indicate that C10 restricts *Mtb* central carbon metabolism. It remains possible that an effect on *Mtb* metabolism is responsible both for the depletion of bacterial
ATP and enhanced sensitivity to INH observed in the presence of C10. However, our earlier findings that the GHTB146 mutant is only resistant to C10-mediated ATP depletion, and not to C10-mediated INH potentiation, suggests that pyruvate limitation is not required for C10 to potentiate INH, since C10 does not deplete pyruvate in this strain. While we have characterized the physiological impact of C10 on *Mtb* metabolism, revealing that pyruvate homeostasis is linked to C10-mediated growth inhibition, these studies stop short of elucidating the target of C10 and uncovering how C10 potentiates killing by INH. Lots of work remains to be done to understand the mechanism of action of C10. This work is worthwhile because uncovering the precise mechanism of action of C10 could reveal vulnerable pathways in *Mtb* that can be exploited to inhibit the bacteria and enhance its sensitivity to clinically relevant antibiotics.

### 4.5 Materials and Methods

**Bacterial strains and growth conditions**

*Mtb* Erdman strains were inoculated from a freezer stock into Middlebrook 7H9 liquid medium supplemented with 60 μL/L oleic acid, 5 g/L BSA, 2 g/L dextrose, 0.003 g/L catalase (OADC), 0.5% glycerol, and 0.05% Tween 80 and cultured at 37°C. Actively growing *Mtb* was then inoculated into Sauton’s liquid medium (0.5 g/L KH₂PO₄, 0.5 g/L MgSO₄, 4.0 g/L L-asparagine, 6% glycerol, 0.05 g/L ferric ammonium citrate, 2.0 g/L citric acid, and 0.01% (wt/vol) ZnSO₄, pH 7.0) supplemented with 0.05% Tween 80 and grown to late-log phase before use in growth curve and survival experiments. For some experiments, modified Sauton’s media was generated with alternative carbon sources or additional supplements, as described in the main text. All carbon sources and supplements were purchased from Sigma. When necessary, the pH of the particular supplements and media was adjusted to pH 7.0 with dropwise of KOH or HCl.
Preparation of compounds

C10 was synthesized using previously described methods \(^{185,250}\) and prepared as an imidazole salt as described previously \(^{45}\). PD217 and SS451 were synthesized using unpublished methods (Anne Mayer Bridwell & Souvik Sarkar, et al. unpublished). Stocks of C10-imidazole were resuspended in DMSO. INH (Sigma) was dissolved in water, and BDQ (Fisher) was dissolved in DMSO. In all experiments, the concentration of both DMSO and imidazole were normalized across all samples to ensure that any differences were due to the effect of the indicated compounds and not due to DMSO or imidazole.

Resazurin assay

Logarithmically growing \(Mtb\) was inoculated into Sauton’s medium in 96 well plates with wells containing increasing concentrations of C10. \(M. tuberculosis\) was inoculated at an OD\(_{600}\) of 0.0025 in 200 \(\mu\)L per well. The plates were incubated at 37°C in 5% CO\(_2\) for 1 week, at which point 32.5 \(\mu\)L of a mixture containing an 8:5 ratio of 0.6 mM resazurin (Sigma) dissolved in 1X phosphate-buffered saline to 20% Tween 80 was added, and the production of fluorescent resorufin was measured on a Synergy HT plate reader with excitation \(\lambda_{ex} = 530\) nm and emission \(\lambda_{em} = 590\) nm after incubation at 37°C in 5% CO\(_2\) overnight. For each assay, medium alone served as a negative control, and untreated \(Mtb\) was included as a positive control. The percent inhibition was calculated as the \(\{[(\text{fluorescence of the positive control} - \text{fluorescence of the negative control}) - (\text{fluorescence of the sample} - \text{fluorescence of the negative control})]/(\text{fluorescence of the positive control} - \text{fluorescence of the negative control})\} \times 100\). When necessary, the IC\(_{50}\) was calculated in GraphPad Prism.

Metabolic profiling by LC-MS

The abundance of metabolites was quantified from extracts of 50mL cultures of \(Mtb\) in Sauton’s
media without Tween 80. For basal metabolite profiling, WT, GHTB136, and GHTB146 were cultured in Sauton’s medium containing 0.05% DMSO and 25μM imidazole for 4 days before quantification. For metabolite profiling from treated Mtb, cultures were exposed to the indicated concentration of C10, BDQ, and/or INH for 3 or 4 days. To extract polar metabolites, the cultures were pelleted, washed twice in H2O, and resuspended in 1.5mL of 2:1 chloroform:methanol in glass conicals. Samples were kept on ice and vortexed each for 1 minute in 20 second intervals, and stored at 4°C overnight. 375μL of H2O was added, the samples were vortexed for 1 minute in 20 second intervals, keeping the samples on ice. Samples were incubated at room temperature for 1 hour with constant agitation, and then centrifuged for 10 minutes at 1000 RPM. The top aqueous layer was transferred to a fresh 1.5mL microcentrifuge tube, stored overnight at -20°C, centrifuged for 5 minutes to pellet any insoluble material, and the supernatant was transferred to a fresh tube. UPLC/MS was performed with an Agilent 1290 Infinity UHPLC system interfaced with an Agilent 6530 QTOF mass spectrometer. Hydrophilic interaction liquid chromatography (HILIC) analysis was performed by using a HILICON iHILIC-(P) Classic column with the following specifications: 100 mm x 2.1 mm, 5 μm. Mobile-phase solvents were composed of A = 20 mM ammonium bicarbonate, 0.1% ammonium hydroxide (adjusted to pH 9.2) and 2.5 μM medronic acid in water:acetonitrile (95:5) and B = 2.5 μM medronic acid in acetonitrile:water (95:5). The column compartment was maintained at 45°C for all experiments. The following linear gradient was applied at a flow rate of 250 μL/min: 0-1 min: 90% B, 1-12 min: 90-35% B, 12-12.5 min: 35-20% B, 12.5-14.5 min: 20% B. The column was re-equilibrated with 20 column volumes of 90% B. The injection volume was 2 μL for all experiments. Data were acquired in both positive and negative ion modes.
4.6 Figures:

**Figure 4.1:** The C10-resistant strain GHTB146 is cross-resistant to new generation C10 analogs. (A) The chemical structures of C10, PD217, and SS451 share a bicyclic 2-pyridone backbone, but the naphthyl moiety of C10 was modified in the newer analogs. (B) WT *Mtb* was incubated in Sauton’s media with the indicated concentrations of C10, PD217, or SS451 for 1 week and the % inhibition of *Mtb* growth and metabolism was determined using the resazurin assay, n=3. (C-D) The indicated strain of *Mtb* was incubated in Sauton’s media with the indicated concentration of PD217 (C) or SS451 (D) and the % inhibition was determined using the resazurin assay, n=3. In B-D the IC<sub>50</sub> values and standard deviations (SD) were calculated in GraphPad Prism and are depicted above the graph. Statistically significant differences were determined by Welch’s one-way ANOVA with Dunnet’s T3 post-test to account for unequal variances across samples, and relevant comparisons are shown. ns not significant, * P<0.05, and ** P<0.01.
Figure 4.2: GHTB146 exhibits basal changes in central carbon metabolism. WT or GHTB146 (designated 146 in the figure) Mtb was cultured in Sauton’s media and polar metabolites were collected from the bacteria and the indicated metabolites were quantified by LC-MS. The fold change in metabolite abundance was calculated relative to the average of the WT samples, and is depicted as a Log2 fold change. The relevant metabolic pathways are depicted, and metabolites in bold type are accompanied with the quantification of the metabolite fold change in GHTB146 compared to WT in the form of a heat map. The heat maps depict 3 biological replicates, with WT in the top row and GHTB146 in the bottom row of each map. Statistically significant differences were determined with an unpaired student’s T test, and all statistically significant differences between GHTB146 and WT are indicated next to the GHTB146 samples. * P<0.05, ** P<0.01. All other comparisons were found not significant (P>0.05).
Figure 4.3: C10 induces metabolic changes in Mtb that are reversed in the C10-resistant mutants GHTB136 and GHTB146. WT, GHTB136, or GHTB146 Mtb was cultured in Sauton’s media containing 25μM C10 or DMSO for 4 days before polar metabolites were collected from the bacteria and the indicated metabolites were quantified by LC-MS. The fold change in metabolite abundance was calculated relative to the average of the DMSO-treated samples within each strain, so metabolic differences between the strains are not depicted. The Log2 fold change was calculated for C10-treated samples normalized to the DMSO control, and is depicted as a heat map. Statistically significant differences were calculated by unpaired student’s T test. We acknowledge the type-1 error inherent in performing multiple student’s T tests. However, a false discovery rate correction resulted in no statistically significant differences among any samples, suggesting that more conservative statistical tests could overlook meaningful biological changes. The P-value (p-val) of the comparison is depicted in a gray-scale heat map next to the samples being compared. ns not significant, * P<0.05, ** P<0.01, and *** P<0.001.
Figure 4.4: The inhibitory activity of Mtb is not dependent on glycerol. (A) WT Mtb was incubated for 1 week in Sauton’s base medium containing 6% glycerol as the primary carbon source, or in modified Sauton’s medium in which the glycerol was omitted and 10mM glucose, 10mM pyruvate, 10mM acetate, 1mM propionate, 10mM α-ketoglutarate, or 10mM glutamate was added as the primary carbon source. Then, the amount of Mtb growth and metabolism was determined using the resazurin assay, where fluorescence is quantified in arbitrary units (AU) as a read-out for increased growth and metabolism. (B) WT Mtb was cultured for 1 week in the presence of the indicated concentration of C10 in Sauton’s base medium containing 6% glycerol as the primary carbon source, or in modified Sauton’s medium in which the glycerol was omitted and 10mM glucose, 10mM pyruvate or 10mM acetate was included as the primary carbon source. The % inhibition was then determined using the resazurin assay, n=3. The IC$_{50}$ values and standard deviations (SD) were calculated in GraphPad Prism and are depicted above the graph. Statistically significant differences were determined in A and B by an ordinary one-way ANOVA with Tukey’s post-test, and relevant comparisons are shown. ns not significant, ** P<0.01, and **** P<0.0001.
Figure 4.5: A targeted chemical rescue screen identifies carbon sources that mitigate the inhibitory activity of C10. WT *Mtb* was incubated in Sauton’s media containing 6% glycerol as the primary carbon source supplemented with the indicated metabolite and exposed to increasing concentrations of C10 for 1 week before the % inhibition of *Mtb* growth and metabolism was determined using the resazurin assay. The IC_{50} of C10 was calculated and is graphed for each condition. The lower dotted line represents the lowest concentration of C10 tested, and therefore the limit of detection (LOD) for the IC_{50} determination. The upper dotted line represents the average IC_{50} of C10 against *Mtb* in the base Sauton’s medium without any additional supplement. Samples that are colored are discussed in the main text. To determine statistically significant difference, the data was Log_{10} transformed to account for the large variation in IC_{50}s across samples and an ordinary one-way ANOVA was performed with Tukey’s post-test. All statistically significant differences are depicted, and any comparison not indicated on the graph was found to be not significant by this test. * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001.
Figure 4.6: Exogenous glucose, pyruvate, acetate, and propionate rescue inhibition by C10 but not BDQ. (A-H) WT Mtb was incubated in Sauton’s media with the indicated concentrations of C10 (A-D) or BDQ (E-H) for 1 week and the % inhibition of Mtb growth and metabolism was determined using the resazurin assay, n=3. Sauton’s media was supplemented with the indicated concentration of glucose (A, E), pyruvate (B, F), acetate (C, G), or propionate (D, H) in addition to the 6% glycerol that is present in the base media. Please note that the same data is used for the no supplement control for C10 in A-D and BDQ in E-H, in order to facilitate comparison between media groups. The IC₅₀ values and standard deviations (SD) were calculated in GraphPad Prism and are depicted above each graph. An ordinary one-way ANOVA with Tukey’s post-test was performed to determine statistically significant differences across samples. Select comparison are depicted in the figure. ns not significant, * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001.
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Figure 4.7: C10 induces a distinct metabolic profile compared to BDQ in the presence and absence of INH. WT Mtb was cultured in Sauton’s media containing 25μM C10, 0.05μg/mL BDQ or DMSO in the presence and absence of 0.25μg/mL INH for 3 days before polar metabolites were collected from the bacteria and the indicated metabolites were quantified by LC-MS. The fold change in metabolite abundance was calculated relative to the average of the DMSO-treated samples within each experiment. The Log2 fold change was calculated for C10-treated samples normalized to the DMSO control and is depicted as a heat map.
Figure 4.8: C10 does not alter NADH/NAD$^+$ homeostasis. WT Mtb was cultured in Sauton’s media containing 25μM C10 in the presence and absence of 0.25μg/mL INH for 3 or 4 days before polar metabolites were collected from the bacteria and NADH and NAD$^+$ levels were quantified by LC-MS. The ratio of NADH/NAD$^+$ was calculated as a metric of the redox homeostasis of this cofactor. To account for the >10-fold difference in the NADH/NAD$^+$ across samples, the data was Log$_{10}$ transformed before performing an ordinary one-way ANOVA, and relevant comparisons are depicted. ns, not significant ****, P<0.0001.
## 4.7 Supplementary Tables

**Supplementary Table 4.S1: Basal metabolite changes in GHTB136 and GHTB146**

The average Log$_2$(Fold Change) in metabolite abundance comparing GHTB136 relative to WT and GHTB146 relative to WT. Fold change data represents the mean of 3 biological replicates. P values were determined as an unpaired student’s t-test comparing the GHTB136 or GHTB146 strain to WT.

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<th>Metabolite</th>
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<th>P (GHTB136 vs WT)</th>
<th>GHTB146 Fold Change</th>
<th>P (GHTB146 vs WT)</th>
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Chapter 5: Identification of 4-amino-thieno[2,3-d]pyrimidines as QcrB inhibitors in Mycobacterium tuberculosis

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Preface:

A version of this chapter was previously published in *mSphere* in 2019. In this chapter some additional data has been added that was omitted from the published manuscript. GAH generated the majority of the data presented in this chapter under the supervision of CLS, with a lot of assistance from co-authors. AEM assisted with some MABA experiments. LAW performed the original screening of a small molecule library that identified CB81 as a lead hit. LAW, JA, KF, SDS, and CLS contributed to screening CB81 analogs in our structure-activity-relationship studies and isolating some early CB81-resistant mutants in *Mycobacterium tuberculosis*. RK and MES assisted with the cloning of ΔcydA and ΔqcrCAB deletion constructs and complementation plasmids. KJ assisted with a number of the experiments using *Mycobacterium smegmatis*. SAW contributed to the molecular modeling of cytochrome *bc*1. Additionally, unless otherwise stated, the compounds used in this study were designed and synthesized by MS and MJM. To clarify our respective contributions throughout chapter, each figure legend will list the initials of the author that generated the data and attribute each panel in the figure to the appropriate contributor.
5.1 Abstract:

Antibiotic resistance is a global crisis that threatens our ability to treat bacterial infections such as tuberculosis, caused by *Mycobacterium tuberculosis* (*Mtb*). Of the 10 million cases of tuberculosis in 2017, approximately 19% of new cases and 43% of previously treated cases were caused by strains of *Mtb* resistant to at least one frontline antibiotic. There is a clear need for new therapies that target these genetically resistant strains. Herein, we report the discovery of a new series of anti-mycobacterial compounds, 4-amino-thieno[2,3-*d*]pyrimidines, that potently inhibit growth of *Mtb*. To elucidate the mechanism by which these compounds inhibit *Mtb*, we selected for mutants resistant to a representative 4-amino-thieno[2,3-*d*]pyrimidine and sequenced these strains to identify the mutations that confer resistance. We isolated a total of 12 resistant mutants, each of which harbored a nonsynonymous mutation in the gene *qcrB*, which encodes a subunit of the electron transport chain (ETC) enzyme cytochrome *bc₁* oxidoreductase, leading us to hypothesize that 4-amino-thieno[2,3-*d*]pyrimidines target this enzyme complex. We found that addition of 4-amino-thieno[2,3-*d*]pyrimidines to *Mtb* cultures resulted in a decrease in ATP levels, supporting our model that these compounds inhibit the *Mtb* ETC. Furthermore, 4-amino-thieno[2,3-*d*]pyrimidines had enhanced activity against a mutant of *Mtb* deficient in cytochrome *bd* oxidase, which is a hallmark of cytochrome *bc₁* inhibitors. Therefore, 4-amino-thieno[2,3-*d*]pyrimidines represent a novel series of QcrB inhibitors that build on the growing number of chemical scaffolds that are able to inhibit the mycobacterial cytochrome *bc₁* complex.

5.2 Significance:

The global tuberculosis (TB) epidemic has been exacerbated by the rise in drug resistant TB cases worldwide. To tackle this crisis, it is necessary to identify new vulnerable drug targets
in *Mycobacterium tuberculosis*, the causative agent of TB, and develop compounds that can inhibit the bacterium through novel mechanisms of action. The QcrB subunit of the electron transport chain enzyme cytochrome *bc*$_1$ has recently been validated as a potential drug target. In this current work, we report the discovery of a new class of QcrB inhibitors, 4-amino-thieno[2,3-*d*]pyrimidines, that potently inhibit *Mtb* growth *in vitro*. These compounds are chemically distinct from previously reported QcrB inhibitors and, therefore, 4-amino-thieno[2,3-*d*]pyrimidines represent a new scaffold that can be exploited to inhibit this drug target.

### 5.3 Introduction:

Infection with *Mycobacterium tuberculosis* (*Mtb*) resulted in over 9 million new cases of tuberculosis (TB) and 1.5 million deaths in 2017, making it the most deadly infectious agent in the world [44]. This epidemic is exacerbated by the rise of multidrug resistant (MDR) TB cases that are resistant to at least the two frontline antibiotics used to treat TB, isoniazid and rifampicin. MDR-TB constituted 3.6% of new TB cases in 2017 and 17% of previously treated TB cases, with rates of MDR-TB as high as 50% of previously treated TB cases in some countries [44]. Furthermore, 8.5% of MDR-TB cases in 2017 were estimated to be extensively drug-resistant (XDR), which are also resistant to a fluoroquinolone and a second-line injectable drug [44]. This rise in drug resistance and scarcity of drugs in the pipeline has made it clear that we are not equipped to successfully battle the ongoing TB epidemic.

In 2012, the diarylquinoline compound bedaquiline (sirturo), which inhibits the mycobacterial ATP synthase [150], was approved to treat MDR-TB patients [149]. The success of this new TB antibiotic fueled interest in mycobacterial energy metabolism pathways as vulnerable targets for new antibiotic development. More recently, the imidazopyridine amide Q203
(telacebec) was identified as a potent antimycobacterial compound that targets QcrB, a subunit of the mycobacterial cytochrome $bc_{1}:aa_{3}$ oxidoreductase in the electron transport chain $^{156}$. Q203 is currently in phase II clinical trials for treatment of TB $^{264}$. Since the discovery of Q203 and additional imidazopyridine amides $^{156,157,265–269}$, a number of compounds have been identified that are also reported to target QcrB, including pyrazolo[1,5-$a$]pyridine-3-carboxamides $^{165,270,271}$, imidazo[2,1-$b$]thiazole-5-carboxamides $^{269}$, pyrrolo[3,4-$c$]pyridine-1,3(2H)-diones $^{158}$, lansoprazole sulfide $^{159}$, 2-(quinolin-4-yloxy)acetamides $^{272,273}$, aryIvinylpiperazine amides $^{160}$, phenoxyalkylbenzimidazoles $^{207,274,275}$, and morpholino thiophenes $^{161}$.

Herein, we present the discovery of 4-amino-thieno[2,3-$d$]pyrimidines as a new series of QcrB inhibitors that potently inhibit $Mtb$ growth and are chemically distinct from previously identified QcrB inhibitors. This work adds to the growing number of QcrB inhibitors that have recently been identified and contributes to our understanding of ways to exploit this target in the development of new chemotherapeutic strategies for TB treatment.

### 5.4 Results:

#### 5.4.1 Identification of 4-amino-thieno[2,3-$d$]pyrimidines with growth inhibitory activity in *Mycobacterium smegmatis* and *Mtb*.

In an effort to identify novel inhibitors of mycobacteria, we screened a selection of 78 small molecule nucleotide mimetics purchased from ChemBridge Corporation for compounds that inhibit growth of $M. \textit{smegmatis}$ in a high throughput liquid culture assay. From these screens we identified a 4-amino-thieno[2,3-$d$]pyrimidine (CB37) that inhibited the growth of $M. \textit{smegmatis}$ (Figure 5.1A-B). We hypothesized that the charged carboxylate group on CB37 may greatly reduce penetration through the cell envelope of the mycobacteria and selected a set of 9 structurally related
compounds without the carboxylate group but containing the 2-ethyl-6-methylthieno[2,3-
d]pyrimidine core (“nearest neighbors”) to purchase and assay for inhibition of *M. smegmatis*
(Figure 5.1, Supplementary Figure 5.S1; Supplementary Table 5.S1, Entries 2-10). Eight of the
compounds either showed similar levels of growth inhibition against *M. smegmatis* as CB37 or no
growth inhibition at all (Supplementary Figure 5.S1). However, one of these compounds, CB81,
showed improved growth inhibition in *M. smegmatis* (Figure 5.1C-D). We resynthesized CB81
and henceforth will designate it as CWHM-728.

To determine if CWHM-728 also had activity in *Mtbc*, we performed zone of inhibition
assays with wild-type (WT) *Mtbc* Erdman strain by spreading approximately 2.5 X 10^8 colony
forming units (CFU) of bacteria on an agar plate and spotting 5 μl of a 100 mM stock of CWHM-
728 dissolved in DMSO onto a disk in the center of the plate. After incubation at 37 °C for 10
days, the bacteria form a lawn and a zone absent of bacterial growth indicates growth inhibition
by the compound. DMSO has no effect on *Mtbc* growth in this assay and does not generate a zone
of clearing on its own, whereas incubation of *Mtbc* with CWHM-728 resulted in growth inhibition
(Figure 5.2A). To test if CWHM-728 has a bacteriostatic or a bactericidal effect on *Mtbc*, we
cultured *Mtbc* in liquid media in the presence of DMSO, 5 μM, or 25 μM CWHM-728, and
enumerated viable CFU after 14 days of incubation (Figure 5.2B). While exposure to 5 μM
CWHM-728 for 14 days caused a slight, but not statistically significant, decrease in CFU
compared to the DMSO-treated control, exposure to 25 μM CWHM-728 caused a significant
reduction in CFU/mL compared to the DMSO control. However, exposure to CWHM-728 did not
decrease the viable CFU below the initial CFU on day 0, indicating that over 14 days, this
concentration of CWHM-728 has a bacteriostatic effect on *Mtbc*.

To identify the half-maximal inhibitory concentration (IC_{50}) of CWHM-728 against *Mtbc*,
we used a microplate Alamar Blue assay (MABA), which is a high-throughput assay commonly used to evaluate the efficacy of anti-mycobacterial compounds \(^{200}\). The MABA utilizes the redox-sensitive dye resazurin, which is blue in its oxidized form but becomes reduced to the pink fluorescent compound resorufin as a result of bacterial metabolism and respiration. Fluorescence can therefore be quantified as a read-out for \(Mtb\) metabolism and respiration, which serves as a proxy for \(Mtb\) growth and/or survival. We incubated \(Mtb\) in the presence of increasing concentrations of CWHM-728 and found that CWHM-728 inhibited \(Mtb\) in the MABA with an IC\(_{50}\) of 2.7 ± 0.84 μM (Figure 5.2C).

To explore chemical modifications that would improve upon the growth-inhibitory activity of CWHM-728 and develop structure-activity relationships (SAR), we used the MABA to test if the analogs that we had previously obtained in which the \(n\)-butyl side chain was replaced with other aliphatic groups had inhibitory activity against \(Mtb\) (Supplementary Table 5.S1). Analogs that have side chains with ionizable groups (entries 1 and 3) and polar groups such as hydroxyl (entry 4) have poor activity, with IC\(_{50}\) values greater than 25 μM. Capping the hydroxyl group as methoxy (entries 5-6) restores some potency, and isopropoxy (entry 7) yields a compound 8-fold more potent than the butyl side chain (entry 2). Cyclopropyl was tolerated (entry 8), with a sub-micromolar IC\(_{50}\), while slightly bulkier cyclopentyl and piperidine side chains resulted in very poor potency (entries 9-10).

To further extend the SAR, we synthesized CWHM-728 analogs containing lipophilic side chains and evaluated their activity in the MABA (Supplementary Table 5.S2). Replacing the \(n\)-butyl side chain with \(t\)-butyl caused a nearly 10-fold decrease in potency (entry 2), but trifluoro-\(n\)-butyl and isopentyl variants (entries 3-4) were found to be greater than 10-fold more potent than the original \(n\)-butyl (entry 1). Capping the NH with a methyl group (entry 5) demonstrated little
effect on potency. Finally, a series of phenyl and alkylphenyl side chains were prepared and showed remarkable SAR with potency being strongly dependent on chain length (entries 6-9).

The most potent compound from our SAR evaluation was CWHM-1023, which shares the 4-amino-thieno[2,3-d]pyrimidine core scaffold with CWHM-728, but contains a 3-phenylpropyl side chain (Figure 5.3A). We found that CWHM-1023 inhibited Mtb growth in the zone of inhibition assay (Figure 5.3B) and inhibited Mtb in the MABA with an IC$_{50}$ of 83 ± 5.4 nM (Figure 5.3C). This is a 38-fold improvement in potency compared to CWHM-728, which had an IC$_{50}$ of 3.2 ± 0.13 μM in this experiment (Figure 5.3C). Thus, CWHM-728 and CWHM-1023 are 4-amino-thieno[2,3-d]pyrimidines that inhibit Mtb at low micromolar and sub-micromolar concentrations, respectively. Following this same trend in potency, we found that the IC$_{50}$ for CWHM-728 and CWHM-1023 in M. smegmatis in the MABA were 52 ± 16 μM and 1.7 ± 0.49 μM, respectively (Figure 5.3D).

5.4.2 Mutations in qcrB confer resistance to 4-amino-thieno[2,3-d]pyrimidines

To identify the target of the 4-amino-thieno[2,3-d]pyrimidines, we selected for Mtb mutants that were resistant to CWHM-728 by plating approximately 2.5 x 10$^8$ CFU of WT Mtb Erdman on 7H11 agar media containing 10μM CWHM-728 and incubating at 37°C for 12 weeks. We found that spontaneous CWHM-728-resistant mutant colonies emerged at an approximate frequency of 1 in 2.3 X 10$^7$. To identify the genetic basis for CWHM-728 resistance, we performed whole genome sequencing on four CWHM-728-resistant strains and found that all four strains harbored missense mutations in the qcrB gene. Three of the strains, isolated from a single culture, had mutations resulting in an A178T amino acid change and one strain, isolated from a second independent culture, had a mutation generating a V338G substitution (Table 5.1). We then sequenced the qcrB locus in 8 additional CWHM-728-resistant mutants isolated from a third
independent culture and identified a missense mutation in \textit{qcrB} in every isolate (Table 5.1). One strain harbored the A178T mutation and 3 more had the V338G mutation that we had isolated previously. In addition, we found that the remaining isolates harbored A178V, G175S, or G315S mutations in \textit{qcrB}.

The \textit{qcrB} gene is located within an operon containing \textit{ctaE}, \textit{qcrC}, and \textit{qcrA}. The \textit{qcrCAB} gene cassette encodes all 3 subunits of the cytochrome \textit{bc}_1 complex \textsuperscript{154}. This complex is localized to the plasma membrane and forms a supercomplex with the \textit{aas}\textsubscript{3}-type cytochrome oxidase encoded by \textit{ctaBCDE} \textsuperscript{276,277}. The resulting cytochrome \textit{bc}_1:aa\textsubscript{3} oxidase complex catalyzes the terminal electron transfer reaction in the mycobacterial electron transport chain (ETC), transferring electrons from menaquinol, the lipid electron carrier in the membrane, to the terminal electron acceptor oxygen. We sought to confirm that mutations in \textit{qcrB} were sufficient to confer resistance to 4-amino-thieno[2,3-d]pyrimidines to rule out the possibility that the CWHM-728-resistant mutants harbored mutations elsewhere in the genome that contributed to resistance. For these studies, we engineered \textit{Mtb} strains that expressed either \textit{qcrCAB}\textsuperscript{WT} or \textit{qcrCAB}\textsuperscript{A178T} from the chromosomal \textit{attB} site and deleted the endogenous \textit{qcrCAB} locus. We then monitored the activity of CWHM-728 (Figure 5.4A) and CWHM-1023 (Figure 5.4B) against these strains using the MABA. We found that the strain expressing \textit{qcrCAB}\textsuperscript{A178T} exhibited reduced sensitivity to both compounds compared to WT \textit{Mtb} or the isogenic \textit{qcrCAB}\textsuperscript{WT} control strain, confirming that this single amino acid change in \textit{qcrB} confers resistance to 4-amino-thieno[2,3-d]pyrimidines. These data are consistent with a model where 4-amino-thieno[2,3-d]pyrimidines target cytochrome \textit{bc}_1.

While it is reportedly difficult to generate \textit{Mtb} mutants deficient for cytochrome \textit{bc}_1, consistent with the growth-inhibitory activity of QcrB inhibitors \textsuperscript{154,167,278}, \textit{ΔqcrCAB} deletion mutants can be generated in \textit{M. smegmatis} \textsuperscript{154}, likely due to the increased respiratory flexibility of
this saprophytic species compared to *Mtb*. We next investigated whether cytochrome *bc₁* expression is required for 4-amino-thieno[2,3-]*d*pyrimidine activity by generating a Δ*qcrCAB* mutant strain of *M. smegmatis*. Using the MABA, we found that CWHM-1023 had reduced inhibitory activity against the *M. smegmatis* Δ*qcrCAB* mutant strain as compared to WT *M. smegmatis* (Fig. 5.4C), supporting that expression of QcrCAB is necessary for full CWHM-1023 activity. Notably, CWHM-1023 still caused inhibition against the Δ*qcrCAB* *M. smegmatis* strain in the MABA, but this activity plateaus at approximately 50% inhibition. This may suggest that CWHM-1023 has off-target effects against *M. smegmatis*, since even in the absence of QcrCAB, CWHM-1023 was able to cause ~50% inhibition against *M. smegmatis* in this assay. Nonetheless, together our data demonstrate that mutations in cytochrome *bc₁* confer resistance to 4-amino-thieno[2,3-*d*]pyrimidines in both *Mtb* and *M. smegmatis*, supporting that these compounds are cytochrome *bc₁* inhibitors.

Based on our finding that mutations in the *qcrB* gene confer resistance to 4-amino-thieno[2,3-*d*]pyrimidines, we propose that this new class of anti-mycobacterial compounds targets QcrB. To better understand how mutations in *qcrB* confer resistance to 4-amino-thieno[2,3-*d*]pyrimidines, we used computational modeling to predict the structure of the *Mtb* QcrB protein based on a previously published structure for QcrB from *Rhodobacter sphaeroides*, which shares 16% amino acid identity with the *Mtb* QcrB 279 (Figure 5.5A). We docked CWHM-728 onto the predicted structure of QcrB and found that it localized near the putative menaquinol binding site of QcrB. Based on our computational model, we speculate that the A178T, A178V, or G175S mutations in QcrB would disrupt contacts required for 4-amino-thieno[2,3-*d*]pyrimidines to bind QcrB (Figure 5.5B). The V338 and G315 residues are not located where they would directly interact with CWHM-728, but are positioned on neighboring helices within a very tightly packed
region such that changes in amino acid identity could possibly affect the position of those helices and disrupt the 4-amino-thieno[2,3-\textit{d}]pyrimidines binding site.

The predicted binding site for the 4-amino-thieno[2,3-\textit{d}]pyrimidines is similar to what has previously been reported from similar modeling studies for the QcrB inhibitor Q203. We investigated whether the 4-amino-thieno[2,3-\textit{d}]pyrimidine resistant strain expressing \textit{qcrCAB}^{A178T} was more resistant to Q203 compared to the isogenic \textit{qcrCAB}^{WT} control strain and found that the \textit{qcrB}^{A178T} mutation did not affect Q203 sensitivity in the MABA (Figure 5.6A). These data suggest that while the \textit{qcrB}^{A178T} allele is sufficient to confer resistance to 4-amino-thieno[2,3-\textit{d}]pyrimidines, it does not confer cross resistance to Q203, possibly because the A187T mutation is not sufficient to disrupt the binding of Q203 to QcrB.

\textbf{5.4.3 4-amino-thieno[2,3-\textit{d}]pyrimidines deplete ATP in \textit{M. smegmatis} and \textit{Mtb}}

Based on our model that 4-amino-thieno[2,3-\textit{d}]pyrimidines target the QcrB subunit of cytochrome \textit{bc}_1, we hypothesized that CWHM-728 and CWHM-1023 inhibit the mycobacterial ETC. To test this hypothesis, we incubated \textit{Mtb} in the presence of 1 \textmu M CWHM-728, 1 \textmu M CWHM-1023, or 400 nM Q203 for 24 hours and measured ATP levels in the bacteria using the BacTiter Glo assay (Promega). We found that treatment with CWHM-728, CWHM-1023, and Q203 decreased ATP levels in \textit{Mtb} by 75\%, 60\%, and 58\%, respectively (Figure 5.6A). Similar results were observed in \textit{M. smegmatis}, where 10 \textmu M CWHM-1023 caused a 41\% decrease in ATP levels in \textit{M. smegmatis} (Figure 5.6B). These data support that 4-amino-thieno[2,3-\textit{d}]pyrimidines target a complex involved in energy generation, consistent with our hypothesis that these compounds inhibit QcrB in the mycobacterial ETC.

\textbf{5.4.4 \textit{M. smegmatis} and \textit{Mtb} lacking cytochrome \textit{bd} are hypersensitive to 4-amino-thieno[2,3-\textit{d}]pyrimidines}
Mycobacteria have a branched ETC that can terminate in either the cytochrome $bc_1:aa_3$ terminal oxidase or in the cytochrome $bd$ terminal oxidase encoded by cydAB$^{154,155}$. Cytochrome $bc_1:aa_3$ oxidase and cytochrome $bd$ oxidase have somewhat overlapping roles in the ETC to transfer electrons from menaquinol to oxygen, where the cytochrome $bd$ oxidase can partially compensate for loss of cytochrome $bc_1:aa_3$ oxidase activity, as evidenced by the observation that mutants of cytochrome $bd$ exhibit increased sensitivity to cytochrome $bc_1$ inhibitors$^{157,166}$. Therefore, we hypothesized that 4-amino-thieno[2,3-d]pyrimidines would exhibit enhanced activity against mycobacteria lacking cytochrome $bd$ as compared with WT strains. To test this hypothesis, we generated $\Delta$cydA $Mtb$ and $\Delta$cydA $M.~smegmatis$ mutants, which lack cytochrome $bd$, and measured the sensitivity of these mutant strains to 4-amino-thieno[2,3-d]pyrimidines as compared to WT strains. We found that deletion of cydA increased the sensitivity of $Mtb$ to CWHM-728 13.8-fold (Figure 5.7A), CWHM-1023 22.6-fold (Figure 5.7B), and Q203 7.7-fold (Figure 5.7C), as measured by a decrease in the MABA IC$_{50}$ compared to WT $Mtb$. Additionally, we found that CWHM-1023 had a 4.6-fold lower IC$_{50}$ against $\Delta$cydA $M.~smegmatis$ compared to WT $M.~smegmatis$, which was partially complemented by expressing cydAB in this strain (Figure 5.7D). These data demonstrate that genetic deletion of cytochrome $bd$ sensitizes mycobacteria to inhibition by 4-amino-thieno[2,3-d]pyrimidines, which is consistent with our hypothesis that these compounds target the QcrB subunit of cytochrome $bc_1$.

5.5. Discussion:

There is a dire need for new anti-TB drugs that shorten treatment regimens and are effective against MDR and XDR strains of $Mtb$. To begin to address this need, two anti-TB agents have recently received accelerated regulatory approval from the United States Food and Drug
Administration (FDA) \(^{149}\). These two drugs, bedaquiline (sirturo) and pretomanid, are the first antibiotics to be approved for the treatment of TB in the last fifty years \(^{281}\). The discovery and development of bedaquiline in particular has raised general interest in targeting \(Mtb\) ETC and respiration as a therapeutic strategy.

Simultaneously, numerous phenotypic screens have identified QcrB inhibitors that inhibit growth of \(Mtb\) in axenic culture and/or in macrophages \(^{156-161,274,282}\), raising further interest in the ETC as a drug target. The most clinically advanced of these QcrB inhibitors is the imidazopyridine amide (IPA) Q203 (telacebec), which is currently in clinical trials for the treatment of TB \(^{264}\). In this study, we report the discovery of a novel class of QcrB inhibitors, 4-amino-thieno[2,3-\(d\)]pyrimidines, that are chemically distinct from the previously identified QcrB inhibitors. Therefore, the new chemical scaffold described herein adds to the growing chemical space that can be exploited to target the mycobacterial cytochrome \(bc_1\) complex. The most potent 4-amino-thieno[2,3-\(d\)]pyrimidine inhibitor of QcrB that we identified, CWHM-1023, had an IC\(_{50}\) of approximately 83 nM in \(Mtb\) in the MABA assay (Table 5.S2). This is in comparison with the known respiration inhibitors Q203, Bedaquiline, and Thiorazidine, which have IC\(_{50}\)s of 1.5 nM, <78 nM, and 11.2 \(\mu\)M against \(Mtb\) in the MABA (Table 5.S2).

Mycobacteria have a branched ETC, whereby electrons can be shuttled to either cytochrome \(bc_1\) or to cytochrome \(bd\) to be transferred to the terminal electron acceptor, \(O_2\) \(^{155}\). Cytochrome \(bc_1\) is proposed to be bioenergetically more efficient than cytochrome \(bd\) under standard aerobic conditions \(^{154}\). However, cytochrome \(bd\) is induced in conditions of low oxygen and contributes to mycobacterial fitness in microaerobic conditions, suggesting that cytochrome \(bd\) can support mycobacterial survival in certain conditions \(^{155}\). As such, while cytochrome \(bc_1\) is important for growth of \(Mtb\) in laboratory conditions \(^{154,283-285}\), \(Mtb\) is able to survive during
inhibition of cytochrome $bc_1^{204,286}$, which is consistent with our data (Figure 5.2B) and previously reported data that QcrB inhibitors lack early bactericidal activity and are bacteriostatic for several weeks $^{160,166,207}$. Furthermore, it was reported that treatment of mutant $Mtb$ lacking cytochrome $bd$ with QcrB inhibitors results in bacterial death $^{160,166}$. Conversely, it was found that overexpression of cytochrome $bd$ in $Mtb$ can enable the bacteria to grow despite cytochrome $bc_1$ inhibition $^{157,278}$. Together, these data indicate that cytochrome $bd$ can compensate for cytochrome $bc_1$ inhibition.

Expression of cytochrome $bc_1$ and cytochrome $bd$ has also been shown to change throughout the course of infection. In mice infected with $Mtb$, it was found that $qcrC$ expression was highest during acute infection (day 15) and following 20 days of infection $qcrC$ was transcriptionally down-regulated 2 to 2.5-fold. In contrast, $cydA$ was transcriptionally up-regulated after 20 days, with the highest expression at 30 days post infection when there was a 7-fold increase over day 15 expression levels $^{168}$. These findings suggest that $Mtb$ may rely more heavily on cytochrome $bc_1$ during acute stages of infection, whereas cytochrome $bd$ is activated at later stages of infection. These expression patterns may explain why it has been found that QcrB inhibitors work well to halt $Mtb$ growth in the mouse when administered during the acute phase of infection $^{156,159,160,165}$, but have a variable impact on bacterial burden when treatment starts after the mice have been infected for more than 2 weeks, with most studies reporting modest decreases in bacterial burden if any decrease at all $^{156,160,165,166}$. Some of the variability of QcrB inhibitor efficacy during the later stages of infection likely also depends on the timing of the treatment and the dosing strategy.

Given the respiratory flexibilty in $Mtb$, interest has increased in using combinations of antibiotics that target different components of the ETC, a strategy that has been shown to lead to synergy in bactericidal effects $^{169,204,287}$. In particular, our studies with 4-amino-thieno[2,3-
\[d\]pyrimidines in the \(\Delta cydA\) mutants support that inhibition of QcrB would have an enhanced impact on \(Mtb\) in combination with inhibitors of cytochrome \(bd\), as previously observed for Q203. Together, these findings support that 4-amino-thieno[2,3-\(d\)]pyrimidines target QcrB, and highlight the idea that concurrent inhibition of both cytochrome \(bc_1\) and cytochrome \(bd\) is more effective than inhibition of cytochrome \(bc_1\) alone. In addition to potentiating other respiration inhibitors, a QcrB inhibitor has recently been shown to enhance pyrazinamide as well as rifampicin monotherapy in mice, indicating that QcrB inhibitors may prove to be a useful addition to the current standard of care.

5.6 Materials and Methods

For more additional information on the methods used in this study that are not included in this section, see section 5.12 Supplementary Materials and Methods.

**Bacterial strains and growth conditions.** \(Mtb\) Erdman was cultured in Middlebrook 7H9 liquid media supplemented with 60 \(\mu\)L/L oleic acid, 5 g/L bovine serum albumin, 2 g/L dextrose, 0.003 g/L catalase (OADC), 0.5% glycerol, and 0.05% Tween 80. For solid media, Middlebrook 7H10 or 7H11 agar media supplemented with OADC and 0.5% glycerol was used. \(M.\) smegmatis mc\(^2\)155 was cultured in LB media supplemented with 0.5% glycerol, 0.5% dextrose, and 0.05% Tween 80. Genetic deletion mutant strains of \(Mtb\) and \(M.\) smegmatis (Table 5.2) were generated using specialized transduction with the conditionally replicating phage phAE87 as previously described. When appropriate, mycobacterial strains were selected on 20 \(\mu\)g/mL kanamycin and/or 50 \(\mu\)g/mL hygromycin.

**Initial Screen.** Logarithmically growing \(M.\) smegmatis strain csm208 was inoculated into 96-well dishes containing 100 \(\mu\)M compounds in 200 \(\mu\)L LB at a starting \(\text{OD}_{600}\) of 0.2 and incubated
shaking at 37°C in a Tecan M200 Pro plate reader with OD_{\lambda_{600}} measurements taken in each well every 15 seconds.

**Microplate Alamar Blue assays (MABAs).** Logarithmically growing *Mtb* was inoculated into 7H9 medium in 96-well plates with wells containing increasing concentrations of compound. *Mtb* was inoculated at an OD_{\lambda_{600}} of 0.0008, corresponding to approximately 4x10^5 CFU/mL in 200 µL per well. Plates were incubated at 37°C in 5% CO\(_2\) for 1 week, at which point 32.5 µL of a mixture containing an 8:5 ratio of 0.6 mM resazurin (Sigma) dissolved in 1X PBS to 20% Tween 80 was added and the production of fluorescent resorufin was measured after incubation at 37°C in 5% CO\(_2\) overnight. For *Mtb*, samples were removed from the plate, and mixed with formalin to kill the *Mtb* before measuring fluorescence. For *M. smegmatis*, the assay plate was measured directly. Fluorescence was measured on a Tecan M200 Pro plate reader with excitation \(\lambda_{ex} = 530\) nm and emission \(\lambda_{em} = 590\) nm. For each assay, media alone served as a negative control and untreated *Mtb* or *M. smegmatis* was included as a positive control. The % inhibition was calculated as the ((positive control - negative control) - (fluorescence of the sample - negative control))/(positive control - negative control) x 100%.

**Selection and Sequencing of Resistant Mutants.** Resistant mutants were selected on 7H11 agar medium containing 10 µM CWHM-728. Approximately 2.5 x 10^8 CFU of WT *Mtb* Erdman was spread per plate and incubated at 37°C for 12 weeks. Genomic DNA was isolated using a CTAB-lysozyme lysis followed by chloroform/isoamyl alcohol extraction and isopropanol precipitation, as previously described \(^{248}\). Whole genome sequencing was performed by Illumina HiSeq with 50 bp single-end reads, and identification of single nucleotide polymorphisms (SNPs) was done using SeqMan NGen (DNASTAR). Genomes were assembled and compared to genomic DNA from the WT parental control strain to identify SNPs that may be responsible for resistance to CWHM-728.
Mutations identified in qcrB by whole genome sequencing were subsequently confirmed by Sanger sequencing. For Sanger sequencing of the qcrB locus, the genomic region was PCR amplified using erdqrBfwxbal430 (GTCTAGAATGAGTCCGAAAACGTGAGTCGCC) and RK27 (GAAGCTTTCCGGGGCTAGTGCTCGCCGTC) and then sequenced using the same two primers (GeneWiz).

**Computational Modeling.** A homology model of *Mtb* QcrB was built using the crystal structure of *Rhodobacter sphaeroides* QcrB (PDB: 2QJP) as the template structure, using the sequence alignment from Ko. The model built in Molecular Operating Environment (MOE 2016.08, Chemical Computing Group, Montreal) was placed into a membrane environment and energy was minimized. The geometry of the [2Fe-2S] center and disulfide bond pattern adjacent to the inhibitor-binding site was assumed to match that of the template structure. CWHM-728 was docked into the homology model using GOLD (v5.5).

**ATP measurements.** *Mtb* or *M. smegmatis* was inoculated into 7H9 medium ± compounds at an OD_{600} of 0.1 and incubated shaking at 37°C for 24 hours (*Mtb*) or 12 hours (*M. smegmatis*). An aliquot of the culture was heat inactivated at 95°C for 20 min and diluted 1:100. Diluted samples were mixed with BacTiter Glo (Promega) reagent at a 1:1 ratio and luminescence was quantified on a Tecan M200 Pro plate reader (integration = 1 second). Relative luminescence units (RLU) were normalized to the log_{10}(CFU) in the sample to account for differences in bacterial number.

**Compounds.** CB37, CWHM-728 (CB81), CWHM-935, CWHM-936, CWHM-941, CWHM-950, CWHM-937, CWHM-946, CWHM-951, and CWHM-942 were purchased from ChemBridge Corporation (www.hit2lead.com). Synthesis of compounds CWHM-1069, CWHM-1020, CWHM-1022, CWHM-1021, CWHM-1304, CWHM-1303, CWHM-1306, and CWHM-1023 is described in section 5.12 Materials and Methods, and LC-MS, \textsuperscript{1}H-NMR, and \textsuperscript{13}C-NMR analysis.
was done on CWHM-1023 to confirm the purity and identity of the synthesized compound (Supplementary Figures 5.S3 and 5.S4). Q203 was acquired from Enamine (EN-300-218150), and bedaquiline (465749185) and thioridazine (1662504) were both purchased from Sigma-Aldrich; all three compounds were tested in the MABA for comparative purposes.

5.7 Acknowledgements

This project was supported by the NIH/National Center for Advancing Translational Sciences (NCATS) grant UL1 TR000448 as well as NIH R33AI111696 and NIH R01AI134847 to C.L.S.. C.L.S. is also supported by a Burroughs Wellcome Fund Investigators in the Pathogenesis of Infectious Disease Award. G.A.H. is supported by a NSF Graduate Research Fellowship DGE-1745038 and the NIGMS Cell and Molecular Biology Training Grant GM007067. K.F. was supported by a pilot award from the Center for Women’s Infectious Disease Research at Washington University. R.L.K. is supported by a Potts Memorial Foundation Postdoctoral Fellowship, J.A. was supported by NIH Grant R25HG006687, M.E.S. was supported through Washington University BioMedRAP, and K.J. was supported through the WUSTL Biology SURF Program. We thank GTAC in the Department of Genetics at WUSTL School of Medicine for help with genomic analysis. The Center is partially supported by NCI Cancer Center Support Grant P30CA91842 to Siteman Cancer Center and by ICTS/CTSA Grant UL1TR000448 from the NCRR and NIH Roadmap for Medical Research.

The authors have no competing financial interests to declare, but acknowledge that C.L.S. has ownership in the company Fimbrion Therapeutics that licenses 4-amino-thieno[2,3-$d$]pyrimidines as QcrB inhibitors in Mycobacterium tuberculosis and, therefore, may financially benefit if the company is successful in marketing its product.
5.8 Figures

Figure 5.1: CB37 and CB81 are 4-amino-thieno[2,3-d]pyrimidines that inhibit growth of *M. smegmatis*. (A) Structure of CB37. (B) *M. smegmatis* (Msm) strain csm208 (Table 5.2) was incubated in LB media in the presence of DMSO, 100 μM CB37, or 73 μM rifampicin (Rif), and the optical density (OD₆₀₀) was measured periodically over the course of 12 hours in a plate reader, n=2. (C) Structure of CB81, which was re-synthesized and re-named CWHM-728. (D) *M. smegmatis* strain csm208 was incubated in LB media in the presence of DMSO, 100 μM CB81, or 73 μM Rif, and the OD₆₀₀ was measured over the course of 12 hours in a plate reader, n=3. All the data in this figure was generated by LAW.
Figure 5.2: CWHM-728 is a 4-amino-thieno[2,3-d]pyrimidine compound that exhibits anti-\textit{Mtb} activity. (A) Zone of inhibition assay was performed by spreading 2.5 X 10^8 CFU of \textit{Mtb} on a plate containing 7H11 agar medium, placing a sterile disk in the center, and pipetting 5 μL of 100% DMSO or 100 mM CWHM-728 on the disk. The plate was incubated at 37ºC for 10 days. Representative image from at least 3 independent experiments is shown. (B) \textit{Mtb} was incubated in 7H9 liquid media in the presence of DMSO or CWHM-728 at the indicated concentrations. At the indicated time points, samples were collected and plated onto 7H11 agar medium containing no antibiotics to enumerate CFU/mL, n=3. Statistical comparisons for the CFU/mL on the final day of the treatment are depicted. *p<0.05, ****p<0.0001, ns = not significant by Two-Way ANOVA with Tukey’s post-test. (C) \textit{Mtb} was incubated with increasing concentrations of CWHM-728 and bacterial respiration and metabolism was measured using the MABA, n=3. The best fit curve and IC_{50} values ± standard deviation were calculated using GraphPad Prism. The data in panels A and B were generated by GAH, and the data in panel C was generated with assistance from AEM.
Figure 5.3: **CWHM-1023 is a 4-amino-thieno[2,3-d]pyrimidine with enhanced activity against mycobacteria.** (A) Chemical structure of CWHM-1023. (B) Zone of inhibition assay with CHWM-1023. 2.5 $\times$ 10$^8$ CFU of *Mtb* was spread on a plate containing 7H11 agar medium, a sterile disk was placed in the center, and 5 $\mu$L of 100 mM CWHM-1023 was pipetted onto the disk. The plate was incubated at 37ºC for 10 days. Representative image from at least 2 independent experiments is shown. (C) *Mtb* or (D) *M. smegmatis (Msm)* was incubated in the presence of increasing concentrations of either CWHM-728 or CWHM-1023 before bacterial respiration and metabolism was measured using the MABA, n=3. Best fit curves and IC$_{50}$ values were calculated using Graphpad Prism. **p<0.01, ****p<0.0001 by student’s t-test. The data in panels B and C were generated by GAH, and the data in panel D was generated with assistance from KJ.
Figure 5.4: Mutation of qcrB confers resistance to 4-amino-thieno[2,3-d]pyrimidines. (A-B) WT or ΔqcrCAB Mtb complemented with either qcrCABWT or qcrCABA178T was incubated in the presence of increasing concentrations of (A) CWHM-728 or (B) CWHM-1023 and then bacterial respiration and metabolism was measured using the MABA, n=3. (C) WT or ΔqcrCAB M. smegmatis (Msm) was incubated in the presence of increasing concentrations of CWHM1023 and bacterial respiration and metabolism was measured using the MABA, n=3. (A-C) Best fit curves and IC₅₀ values ± standard deviation (S.D.) were calculated using GraphPad Prism. No IC₅₀ estimate is included for panel C because the ΔqcrCAB curve plateaus around 50% inhibition. ns = not significant, *p<0.05 by student’s t-test. The data in panels A and B were generated by GAH, and the data in panel C was generated with assistance from KJ.
Figure 5.5: Molecular modeling of CWHM-728 in predicted structure of *Mtb* QcrB. (A) QcrB homology model embedded in lipid bilayer, with lipid headgroup phosphorus as magenta spheres. (B) QcrB binding site shown with CHWM-728 docked (green). Residues mutated in resistance mutants are highlighted in magenta. The model in this figure was generated by SAW.
Figure 5.6: 4-amino-thieno[2,3-d]pyrimidines deplete mycobacterial ATP levels. (A) *Mtb* was incubated in the presence of 1 μM CWHM-728, 1 μM CWHM-1023, or 400 nM Q203 for 24 hours, the samples were heat-inactivated, and ATP was quantified using BacTiter Glo. (B) *M. smegmatis* (*Msm*) was incubated in the presence of 10 μM CWHM-1023 for 12 hours and ATP was quantified using BacTiter Glo. In both A and B, ATP levels were normalized to the log10(CFU) to account for differences in bacterial numbers due to differences in growth. ***p<0.001, ****p<0.0001, ns = not significant by (A) One-Way ANOVA with Tukey’s post-test or (B) student’s t-test. The data in this figure was generated by GAH.
Figure 5.7: Mycobacteria lacking cytochrome bd have increased sensitivity to 4-aminothieno[2,3-d]pyrimidines. (A-C) Either WT or ΔcydA Mtb was incubated in the presence of increasing concentrations of (A) CWHM-728, (B) CWHM-1023, or (C) Q203 and respiration and metabolism was measured using the MABA, n=3. (D) WT M. smegmatis (Msm) or ΔcydA Msm complemented with empty vector (EV), or with a plasmid harboring cydAB were incubated in the presence of increasing concentrations of CWHM-1023 and respiration and metabolism were measured using the MABA, n=3. (A-D) Best fit curves and IC₅₀ values ± standard deviation (S.D.) were generated using GraphPad Prism. *p<0.05, ****p<0.0001, ns = not significant by (A-C) student’s t-test or (D) One-Way ANOVA with Tukey’s post-test. The data in panels A, B, and C were generated by GAH, and the data in panel D was generated with the assistance of KJ.
### 5.9 Tables

**Table 5.1: CWHM-728-resistant isolates harbor mutations in qcrB.**

CWHM-728-resistant strains isolated in this study are listed with the mutation identified in *qcrB*. The strains were isolated from three independent cultures, designated by a, b, or c in superscript.

*Mutations identified by whole genome sequencing. All other mutations were identified by Sanger sequencing of the *qcrB* locus.

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5.10 Supplementary Figures

Supplementary Figure 5.S1: Related 4-amino-thieno[2,3-d]pyrimidines do not inhibit growth of *M. smegmatis* greater than CB37. *M. smegmatis* strain csm208 was incubated in the presence of the indicated 4-amino-thieno[2,3-d]pyrimidine at 100 μM and the optical density (OD) was measured periodically over the course of 12 hours in a plate reader, n=3. (A-F) DMSO and 73 μM rifampicin were included as controls. The control data for A-F are the same in each panel because all the compounds were tested in the same assay plate. (G-H) DMSO and 1.1 mM streptomycin were included as controls. The control data for G-H are the same in both panels because these compounds were tested in the same assay plate. The data in this figure was generated by LAW.
Supplementary Figure 5.S2: *Mtb* harboring the *qcrB<sup>A178T</sup>* allele are not cross-resistant to Q203. WT or ΔqcrCAB *Mtb* complemented with either *qcrCAB<sup>WT</sup>* or *qcrCAB<sup>A178T</sup>* was incubated in the presence of increasing concentrations of Q203 and then bacterial respiration and metabolism was measured using the MABA, n=3. Best fit curves and IC<sub>50</sub> values ± standard deviation (S.D.) were calculated using GraphPad Prism. *p<0.05, **p<0.01, ns = not significant by One-Way ANOVA with Tukey’s post-test. The data in this figure was generated by GAH.
Supplementary Figure 5.S3: LC-MS spectra for CWHM-1023. CWHM-1023 was subjected to LC-MS analysis as described in the supplementary information. The data in this figure was generated by MS and MJM.
Supplementary Figure 5.S4: NMR spectra for CWHM-1023. CWHM-1023 was subjected to (A) $^1$H-NMR and (B) $^{13}$C-NMR analysis. The inset in panel A depicts the chemical structure of CWHM-1023. The data in this figure was generated by MS and MJM.
5.11 Supplementary Tables

Supplementary Table 5.51: Structure-Activity Relationship Studies Around CB37

\(^a\) clogP = calculated log(partition coefficient, P). Values were calculated using CDD Vault ([www.collaborativemedicine.com](http://www.collaborativemedicine.com)). \(^b\) Values from Figure 5.2C.

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Supplementary Table 5.S2: Structure-Activity Relationship Studies around CWHM-728 and comparison to known *Mtb* inhibitors

\(^a\) clogP = calculated log(partition coefficient, P). Values were calculated using CDD Vault ([www.collaborativedrug.com](http://www.collaborativedrug.com)). \(^b\)Values from Figure 5.3C.

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5.12 Supplementary Materials and Methods

Chemical Synthesis: Instrumentation and General Methods.

The starting materials were obtained from commercial sources and used without further purification after verifying their purities by LC-MS analysis. Reactions were performed under an atmosphere of dry nitrogen unless otherwise stated. Solvents were analytical grade and used as supplied. Analytical HPLC analyses were performed on an Agilent 1100 system and LC-MS analyses were conducted on Agilent 1100 Series LC/MSD (G1946C) single quadrupole mass spectrometer system equipped with an electrospray ionization (ESI) source. Reverse-phase preparative HPLC purifications were performed either on a Biotage SP4 HPFC system or on a CombiFlash\textit{Rf} (Teledyne Isco) system using a variable dual wavelength UV detector on a Biotage KP-C18-HS 120 g SNAP column and on Redisep Rf Gold C18 cartridges using acetonitrile/water gradient containing 0.05% TFA. Normal phase preparative HPLC purifications were performed either on a Biotage SP4 HPFC system or on a CombiFlash\textit{Rf} (Teledyne Isco) system using a variable dual wavelength UV detector with pre-packed Biotage KP-SIL SNAP cartridges and Redisep Rf silica gel (Isco) cartridges and ethyl acetate/hexanes gradients.

All final compounds were analyzed by analytical HPLC using a C18 analytical column with a diode array detector and peaks were monitored at 210, 254 and 280 nM for their purity. $^1$H and $^{19}$F NMR spectra were recorded in deuterated solvents (DMSO-$d_6$, CD$_3$OD and CDCl$_3$) on a Bruker \textit{Avance}-III/400 MHz spectrometer equipped with a Broad Band NMR probe. The signal of the deuterated solvent was used as an internal reference. The chemical shifts are expressed in ppm ($\delta$) and coupling constants ($J$) are reported in hertz (Hz). Samples were analyzed by high resolution mass spectrometry using flow injection analysis coupled to a Q-Exactive mass spectrometer.
Samples were injected at 50µl/min with 90% ACN, 10% water with 0.1% formic acid. The electrospray voltage was set to 1,500 V and the accuracy of the instrument was determined to be less than 5 ppm.

**Synthesis of thieno[2,3-d]pyrimidine analogs.**

The general procedures described by Prasad and coworkers were modified as described below.

![Chemical structure of synthetic pathway](image)

**2-Ethyl-6-methyl-3H-thieno[2,3-d]pyrimidin-4-one (2).** A dark red mixture of 2-amino-3-ethoxycarbonyl-5-methylthiophene (1; 648.0 mg, 3.5 mmol) and propionitrile (0.5 mL, 7.0 mmol) was treated with 4.0 M HCl in 1,4-dioxane (3 mL, 12.0 mmol) at room temperature. The reaction mixture quickly turned to a thick yellow-orange paste. An additional 2 mL 4N HCl in 1,4-dioxane was added after 30 min and the reaction mixture was heated at 50 °C to give a red solution. LC-MS analysis of the reaction mixture after 1.5 h showed the uncyclized intermediate product and the intermediate's mass: m/z 241 [M+H]^+; no traces of the starting materials were present. After heating at 50 °C for 2 h, the reaction mixture was heated at 110 °C to give a dark red solution. A thick yellow paste began to form within 1 h. The reaction mixture was heated at 110 °C overnight. The solvent was evaporated in vacuo to afford a yellow-brown solid. The solid was dissolved in acetonitrile (20 mL) and cooled to room temperature to afford a crystalline precipitate. The solid was filtered, washed with acetonitrile (2×10 mL) and dried in vacuo to give a cream crystalline solid (648.0 mg, yield 95%). LC-MS purity >98%; m/z 195 [M+H]^+ and 217 [M+Na]^+. ^1H NMR (400 MHz, DMSO-d6): δ 1.20 (t, J = 7.58 Hz, 3H), 2.47 (d, J = 1.22 Hz, 3H), 2.61 (q, J =7.58 Hz, 2H), 7.02 (d, J = 1.22 Hz, 1H), 11.59 (br. s, 1H).
4-Chloro-2-ethyl-6-methylthieno[2,3-d]pyrimidine (3).

A suspension of 2-ethyl-6-methyl-3H-thieno[2,3-d]pyrimidin-4-one (648.0 mg, 3.34 mmol) in phosphorus oxychloride (4 mL, 42.91 mmol)) was heated at refluxing conditions. Within 1 h a light brown-orange solution was obtained. The solvent was evaporated in vacuo to afford a light orange-brown viscous liquid. The liquid was poured onto crushed ice-water to give a cream precipitate. The mixture was neutralized with a saturated NaHCO₃ solution to give a cream precipitate. The precipitate was extracted with ethyl acetate (2×25 mL), the aqueous and the organic layers were separated, the organic layer was washed with brine (1×25 mL) and dried over anhydrous Na₂SO₄, filtered and evaporated in vacuo to afford a very light brown liquid which solidified to a cream crystalline solid (620.0 mg, yield 88%). LC-MS purity >98%; m/z 213 [³⁵Cl⁺M+H]⁺ and 215 [³⁷Cl⁺M+H]⁺. ¹H NMR (400 MHz, DMSO-d₆): δ 1.30 (t, J = 7.55 Hz, 3H), 2.63 (d, J = 1.34 Hz, 3H), 2.96 (q, J = 7.54 Hz, 2H), 7.23 (d, J = 1.34 Hz, 1H).

2-Ethyl-6-methyl-N-(4,4,4-trifluorobutyl)thieno[2,3-d]pyrimidin-4-amine (CWHM-1020).

A solution of 4-chloro-2-ethyl-6-methyl-thieno[2,3-d]pyrimidin (50 mg, 0.235 mmol), DIEA (50 µL, 0.292 mmol) and 4,4,4-trifluorobutylamine (66 µL, 0.705 mmol) in 1,4-dioxane was heated to 140 °C for 60 min in a microwave reactor. The reaction mixture was partitioned between water and DCM. The DCM layer was separated and concentrated and the crude product was purified by reverse phase HPLC to give the desired product. The residue was dissolved in acetonitrile containing a couple drops of methanol and eluted through a SiliaPrep Carbonate 6mL-1g plug. Evaporation of the solvent in vacuo afforded the product as a white solid (26.1 mg, yield 37%). LC-MS purity >98%; m/z 304 [M + H]⁺. ¹H NMR (400 MHz, DMSO-d₆): δ 7.68 (t, J = 5.53 Hz, 1H), 7.18 (d, J = 1.34 Hz, 1H), 3.53 (q, J = 6.66 Hz, 2H), 2.67 (q, J = 7.56 Hz, 2H), 2.49 (d, J = 1.00 Hz, 3H), 2.24 - 2.44 (m, 2H), 1.76 - 1.90 (m, 2H), 1.22 (t, J = 7.55 Hz, 3H); ¹⁹F NMR (376
MHz, DMSO-\textit{d}_6): \delta -64.68. \textit{HRMS (ESI)} m/z: [M + H]\textsuperscript{+} \textit{Calcd for C}_{13}\textsubscript{H}_{17}\textsubscript{F}_{3}\textsubscript{N}_{3}S 304.1090; found 304.1078.

\textit{N-Butyl-2-ethyl-N,6-dimethylthieno[2,3-d]pyrimidin-4-amine (CWHM-1021).}

A solution of 4-chloro-2-ethyl-6-methyl-thieno[2,3-d]pyrimidine (50 mg, 0.235 mmol), DIEA (50 \mu L, 0.292 mmol) and \textit{N}-methyl-\textit{n}-butylamine (84 \mu L, 0.705 mmol) in 1,4-dioxane was heated to 140 °C for 60 min in a microwave reactor. The reaction mixture was partitioned between water and DCM. The DCM layer was separated and concentrated and the crude product was purified by reverse phase HPLC to give the desired product. The residue was dissolved in acetonitrile containing a couple drops of methanol and eluted through a SiliaPrep Carbonate 6mL-1g plug to neutralize. Evaporation of the solvent \textit{in vacuo} afforded as an oil which was dissolved in acetonitrile/water and lyophilized to give a white solid (24.8 mg, yield 40%). \textit{LC-MS purity >98%; m/z 264 [M + H]\textsuperscript{+}.} \textit{\textit{1}H NMR (400 MHz, DMSO-\textit{d}_6): \delta 7.24 (d, J = 1.28 Hz, 1H), 3.66 - 3.77 (m, 2H), 3.32 (s, 3H), 2.66 (q, J = 7.58 Hz, 2H), 2.49 (d, J = 1.16 Hz, 3H), 1.55 - 1.65 (m, 2H), 1.27 - 1.39 (m, 2H), 1.23 (t, J = 7.58 Hz, 3H), 0.92 (t, J = 7.37 Hz, 3H).} \textit{HRMS (ESI)} m/z: [M + H]\textsuperscript{+} \textit{Calcd for C}_{14}\textsubscript{H}_{22}\textsubscript{N}_{3}S 264.1529; found 264.1519.

\textit{2-Ethyl-6-methyl-N-(3-methylbutyl)thieno[2,3-d]pyrimidin-4-amine (CWHM-1022).}

A solution of 4-chloro-2-ethyl-6-methyl-thieno[2,3-d]pyrimidine (50 mg, 0.235 mmol), DIEA (50 \mu L, 0.292 mmol) and isopentylamine (82 \mu L, 0.705 mmol) in 1,4-dioxane was heated to 140 °C for 60 min in a microwave reactor. The reaction mixture was partitioned between water and DCM. The DCM layer was separated and concentrated and the crude product was purified by reverse phase HPLC to give the desired product. The residue was dissolved in acetonitrile containing a couple drops of methanol and eluted through a SiliaPrep Carbonate 6mL-1g plug to neutralize TFA. Evaporation of the solvent \textit{in vacuo} afforded the product as a white solid (29.0 mg, yield
47%). LC-MS purity >98%; \textit{m/z} 264 [M + H]+. \(^1\)H NMR (400 MHz, DMSO-\textit{d}$_6$): \(\delta\) 7.54 (t, \(J = 5.44\) Hz, 1H), 7.19 (d, \(J = 1.22\) Hz, 1H), 3.42 - 3.55 (m, 2H), 2.66 (q, \(J = 7.58\) Hz, 2H), 2.48 (s, 3H), 1.63 (td, \(J = 6.65, 13.36\) Hz, 1H), 1.48 (q, \(J = 6.85\) Hz, 2H), 1.22 (t, \(J = 7.58\) Hz, 3H), 0.92 (d, \(J = 6.54\) Hz, 6H). HRMS (ESI) \textit{m/z}: [M + H]+ Calcd for C$_{14}$H$_{22}$N$_3$S 264.1529; found 264.1518.

2-Ethyl-6-methyl-N-(3-phenylpropyl)thieno[2,3-\textit{d}]pyrimidin-4-amine (CWHM-1023).

A solution of 4-chloro-2-ethyl-6-methylthieno[2,3-\textit{d}]pyrimidine (50 mg, 0.235 mmol), DIEA (50 µL, 0.292 mmol) and 3-phenyl-n-propylamine (100 µL, 0.705 mmol) in 1,4-dioxane was heated to 140 °C for 60 min in a microwave reactor. The reaction mixture was partitioned between water and DCM. The DCM layer was separated and concentrated and the crude product was purified by reverse phase HPLC to give the desired product. The residue was dissolved in acetonitrile containing a couple drops of methanol and eluted through a SiliaPrep Carbonate 6mL-1g plug to neutralize TFA. Evaporation of the solvent \textit{in vacuo} afforded the product as a white solid (32.1 mg, yield 44%). LC-MS purity >98%; \textit{m/z} 312 [M + H]+ (Figure 5.S3). \(^1\)H NMR (400 MHz, DMSO-\textit{d}$_6$): \(\delta\) 7.64 (t, \(J = 5.47\) Hz, 1H), 7.12 - 7.35 (m, 6H), 3.44 - 3.54 (m, 2H), 2.67 (qd, \(J = 3.79, 11.37\) Hz, 4H), 2.50 (d, \(J = 1.16\) Hz, 3H), 1.86 - 1.99 (m, 2H), 1.22 (t, \(J = 7.58\) Hz, 3H) (Figure 5.S4A). \(^{13}\)C NMR (100 MHz, CDCl$_3$) \(\delta\) 166.7, 156.0, 141.6, 136.2, 128.5, 128.4, 126.0, 114.4, 114.2, 40.7, 33.4, 32.1, 16.3, 12.8 (Figure 5.S4B). HRMS (ESI) \textit{m/z}: [M + H]+ Calcd for C$_{18}$H$_{22}$N$_3$S 312.1529; found 312.1517.

\textit{N}-tert-Butyl-2-ethyl-6-methyl-thieno[2,3-\textit{d}]pyrimidin-4-amine (CWHM-1069).

A solution of 4-chloro-2-ethyl-6-methyl-thieno[2,3-\textit{d}]pyrimidine (60.5 mg, 0.28 mmol), DIEA (100 µL, 0.58 mmol) and \textit{tert}-butylamine (90 µL, 0.85 mmol) in 1,4-dioxane (1 mL) was heated at from 140 to 200 °C in a microwave reactor for several hours (>18 h) to give a yellow-orange solution. The solvent was evaporated \textit{in vacuo} to give a light tan crystalline solid and the crude
residue was purified by reverse-phase preparative HPLC to afford a colorless to cream crystalline residue. The purified residue was dissolved in acetonitrile containing a trace of methanol and the solution was passed through a SiliaPrep Carbonate (Si-CO\(_3\)) 6 mL-1 g cartridge. The filtrate was evaporated \textit{in vacuo} to afford a colorless powder (37.3 mg, yield 53%). LC-MS purity >98%; \(m/z\) 250 [M + H]\(^+\). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 1.24 (t, \(J = 7.58\) Hz, 3H), 1.50 (s, 9H), 2.48 (d, \(J = 1.22\) Hz, 3H), 2.68 (q, \(J = 7.58\) Hz, 2H), 6.82 (s, 1H), 7.37 (d, \(J = 1.22\) Hz, 1H). HRMS (ESI) \(m/z\): [M + H]\(^+\) Calcd for C\(_{13}\)H\(_{20}\)N\(_3\)S 250.1372; found 250.1362.

\textit{N-Benzyl-2-ethyl-6-methylthieno[2,3-d]pyrimidin-4-amine (CWHM-1303).}

A solution of 4-chloro-2-ethyl-6-methyl-thieno[2,3-d]pyrimidine (67.0 mg, 0.315 mmol), DIEA (110 \(\mu\)L, 0.643 mmol) and benzylamine (105 \(\mu\)L, 0.96 mmol) in 1,4-dioxane (2 mL) was heated at 140 °C in a microwave reactor for 3 h to give a pale yellow solution. The solvent was evaporated \textit{in vacuo} to give a cream crystalline solid. The crude residue was purified by reverse-phase preparative HPLC to afford a colorless viscous liquid containing a colorless crystalline solid (130.3 mg). The purified residue was dissolved in acetonitrile containing a trace of methanol and the solution was passed through a SiliaPrep Carbonate (Si-CO\(_3\)) 6 mL-1 g cartridge to neutralize TFA. The filtrate was evaporated \textit{in vacuo} to afford a colorless crystalline solid (90.0 mg, yield 100%). LC-MS purity >98%; \(m/z\) 284 [M + H]\(^+\). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 1.37 (t, \(J = 7.58\) Hz, 3H), 2.53 (s, 3H), 2.87 (q, \(J = 7.58\) Hz, 2H), 4.84 (d, \(J = 5.62\) Hz, 2H), 5.17 (br. s, 1H), 6.71 (s, 1H), 7.28-7.37 (m, 3H), 7.38-7.42 (m, 2H). HRMS (ESI) \(m/z\): [M + H]\(^+\) Calcd for C\(_{16}\)H\(_{18}\)N\(_3\)S 284.1216; found 284.1205.

\textit{2-Ethyl-6-methyl-N-phenylthieno[2,3-d]pyrimidin-4-amine (CWHM-1304).}

A solution of 4-chloro-2-ethyl-6-methyl-thieno[2,3-d]pyrimidine (71.6 mg, 0.34 mmol), DIEA (120 \(\mu\)L, 0.70 mmol) and aniline (95 \(\mu\)L, 1.04 mmol) in 1,4-dioxane (2 mL) was heated at 200 °C
in a microwave reactor for 6 h to give a dark yellow solution. The solvent was evaporated in vacuo to give a pale yellow viscous liquid solidified slowly to a dirty cream crystalline solid (176.6 mg). The crude residue was purified by reverse-phase preparative HPLC to afford a colorless crystalline precipitate in water. The solid was filtered, washed with water (3×10 mL) and dried in vacuo to afford a colorless solid. The purified residue was dissolved in acetonitrile containing a trace of methanol and the solution was passed through a SiliaPrep Carbonate (Si-CO₃) 6 mL-1 g cartridge to neutralize TFA. The filtrate was evaporated in vacuo to afford a cream crystalline solid (88.9 mg; yield 98%). LC-MS purity >98%; m/z 270 [M + H]⁺. ¹H NMR (400 MHz, CDCl₃): δ 1.41 (t, J = 7.58 Hz, 3H), 2.56 (s, 3H), 2.94 (q, J = 7.58 Hz, 2H), 6.73 (s, 1H), 6.80 (br. s, 1H), 7.14 (t, J = 7.45 Hz, 1H), 7.39 (t, J = 7.70 Hz, 2H), 7.71 (d, J = 8.31 Hz, 2H). HRMS (ESI) m/z: [M + H]⁺ Calcd for C₁₅H₁₆N₃S 270.1059; found 270.1048.

2-Ethyl-6-methyl-N-phenethylthieno[2,3-d]pyrimidin-4-amine (CWHM-1306).

A solution of 4-chloro-2-ethyl-6-methyl-thieno[2,3-d]pyrimidine (67.0 mg, 0.32 mmol), DIEA (110 µL, 0.64 mmol) and 2-phenylethylamine (119 µL, 0.95 mmol) in 1,4-dioxane (2 mL) was heated at 140 °C in a microwave reactor for 3 h to give a colorless solution and the solvent was evaporated in vacuo to give a pale viscous liquid. The crude residue was purified by reverse-phase preparative HPLC to afford a colorless crystalline solid (132.0 mg). The purified residue was dissolved in acetonitrile containing a trace of methanol and the solution was passed through a SiliaPrep Carbonate (Si-CO₃) 6 mL-1 g cartridge. The filtrate was evaporated in vacuo to afford a colorless crystalline solid (90.0 mg, yield 96%). LC-MS purity >98%; m/z 298 [M + H]⁺. ¹H NMR (400 MHz, CDCl₃): δ 1.39 (t, J = 7.58 Hz, 3H), 2.53 (s, 3H), 2.87 (q, J = 7.58 Hz, 2H), 2.99 (t, J = 6.97 Hz, 2H), 3.89 (q, J = 6.60 Hz, 2H), 4.95 (br. s, 1H), 6.60 (s, 1H), 7.23-7.29 (m, 3H), 7.32-7.39 (m, 2H). HRMS (ESI) m/z: [M + H]⁺ Calcd for C₁₇H₂₀N₃S 298.1372; found 298.1361.
Chapter 6: Conclusions and Future Directions

Gregory A Harrison
6.1 Major findings regarding the activities of C10

6.1.1 C10 potentiates killing by INH and can re-sensitize \textit{katG} mutants to inhibition by INH

By screening for small molecules that inhibit biofilm formation, rather than for compounds that simply inhibit \textit{Mtb} growth, we identified the bicyclic 2-pyridone compound C10, which inhibited biofilm formation and had poor growth-inhibitory activity on its own (Figure 2.1). When we treated \textit{Mtb} with C10 in hypoxic conditions, an environment that enriches for stress and drug tolerant cells \textsuperscript{30}, we found that C10 sensitized hypoxic \textit{Mtb} to killing by H\textsubscript{2}O\textsubscript{2} and INH (Figure 2.1), suggesting that C10 can make tolerant bacteria susceptible to killing by oxidative stress or INH challenge. However, C10 did not block pan-drug tolerance in this condition, as C10 did not sensitize the hypoxic bacteria to killing by ethambutol, rifampicin, or kanamycin (Supplementary Figure 2.S2). Therefore the effects of C10 are specific for INH and not other antibiotics. We subsequently found that C10 can sensitize \textit{Mtb} to killing by INH in aerobic conditions as well (Figure 2.2), suggesting that this effect is not limited to the hypoxia model.

We showed that C10 was able to prevent the emergence of INH-resistant mutants \textit{in vitro} (Figure 2.2). In our study, every INH-resistant mutant that we checked by sequencing harbored a mutation in \textit{katG}. One explanation for how C10 suppresses the selection for these mutants in the presence of INH is that C10 could be toxic to mutants lacking \textit{katG}. There is some evidence to support this possibility, as the IC\textsubscript{50} of C10 against some \textit{katG} mutants is 1.6-2.9-fold lower than its IC\textsubscript{50} against WT (Figure 3.3). However, when we take these very same INH-resistant \textit{katG} mutants and culture them in liquid media containing C10 or spread them on agar media that contains C10, we find that these strains can still grow in the presence of C10 (Figure 2.3 and Figure
Therefore, growth inhibition by C10 alone is not sufficient to explain why these mutants fail to emerge on agar containing INH and C10. Instead, we demonstrated that C10 is able to re-sensitize several of these mutants to inhibition in the presence of INH (Figure 2.3 and Figure 3.3), indicating that treatment with C10 and INH together can somehow circumvent INH resistance in these mutants.

6.1.2 C10 disrupts *Mtb* metabolism and/or respiration

To understand how C10 has these effects, we performed transcriptional profiling on C10-treated *Mtb* and found that C10 induces a transcriptional signature consistent with inhibition of energy metabolism (Figure 2.4). We subsequently demonstrated that C10 decreases *Mtb* O$_2$ consumption, sensitizes the bacteria to killing by the ETC inhibitor Q203, and sensitizes the bacteria to killing in low pH, which are all consistent with disruptions in energy homeostasis (Figure 2.4, Figure 2.5, and Figure 2.6). Furthermore, exposure to C10 decreases *Mtb* ATP levels by approximately 50%, which is a hallmark of inhibitors that target *Mtb* energy metabolism (Figure 2.4, Figure 3.2, and Figure 3.3). These findings led us to wonder if the inhibition of energy metabolism by C10 was driving the enhanced susceptibility to INH.

Since our studies had still not uncovered the target and mechanism of action of C10, we sought to take a forward genetic approach to isolate mutants resistant to C10, in the hopes of identifying genes linked to the mechanism of action of C10. Up until this point, we had been using a sub-inhibitory concentration of C10 (5-25μM) that caused only a modest decrease in the *Mtb* growth rate (Figure 2.2 and Figure 3.3). By increasing the concentration, we demonstrated that higher concentrations of C10 cause a dose-dependent decrease in bacterial growth (Figure 3.1). At the highest concentration tested, 200μM, C10 caused complete growth inhibition, and we were able to isolate resistant mutants that could grow on this concentration of C10. High-level C10
resistance was demonstrated in 2 strains that up-regulate either a putative SAM-methyltransferase
\( (Rv0731c) \) or the \( lpdA-glpD2 \) operon (Figure 3.1 and Supplementary Figure 3.S1). Further
investigation is still needed to precisely understand how these mutations confer C10 resistance.
However, in both strains, we observed that C10 failed to efficiently deplete bacterial ATP (Figure
3.1), suggesting they are resistant to the effects of C10 on \( Mtb \) energy metabolism. Additionally,
this finding indicates that C10 toxicity is connected to bacterial ATP depletion.

6.1.3 Depletion of bacterial ATP by C10 is neither required nor sufficient to
potentiate INH

Using these C10-resistant mutants, we were able to test whether the effects of C10 on
energy metabolism are required for C10 to potentiate killing by INH, since the ability of C10 to
deplete ATP is lost in these mutants. By culturing these strains in the presence of C10 and/or INH
and monitoring survival, we found that C10 still enhanced the bactericidal activity of INH against
these strains to a similar or even greater extent compared to WT \( Mtb \) (Figure 3.2). Therefore, the
ability of C10 to deplete ATP is not required for C10 to potentiate killing by INH. In a
complementary approach, I used the direct ATP synthase inhibitor BDQ to deplete bacterial ATP
to a similar magnitude as C10 and demonstrated that ATP depletion with BDQ did not potentiate
killing by INH (Figure 3.2). Instead, cultures exposed to both BDQ and INH harbored more
surviving bacteria than INH alone, suggesting that BDQ actually antagonizes killing by INH.
These findings are consistent with published data in the field that ETC inhibitors such as BDQ,
Q203, and CCCP antagonize the bactericidal activity of INH \(^{120-122}\). Therefore, depletion of ATP
is neither required nor sufficient to potentiate INH. Instead, our findings with C10 challenge the
paradigm in the field that inhibitors of \( Mtb \) ATP synthesis antagonize killing by INH \(^{19,120-122}\). C10
has the remarkable ability to potentiate killing by INH in spite of inhibiting energy metabolism.
and decreasing bacterial ATP. C10 must impact a pathway that sensitizes the bacteria to INH despite the decreased ATP levels.

### 6.1.4 C10 sensitizes *Mtb* to INH-NAD without impacting its activation by making bacteria vulnerable to InhA inhibition

To more specifically understand how C10 potentiates killing by INH, we took a second forward genetic approach to isolate resistant mutants in the context of both C10 and INH. Through this approach, we isolated a strain that was resistant to INH even in the presence of C10 (Figure 3.3). This mutant harbored a single point mutation in the genome leading to an early stop in the *katG* coding region. We were initially perplexed by this finding but found that the previous *katG* mutants we had studied all retained some residual KatG activity, and were actually hypomorphic for *katG*, exhibiting decreased expression or expressing a KatG variant with decreased activity (Figure 3.4). In contrast, the new *katG* mutant we isolated was completely null for KatG activity (Figure 3.4), suggesting that some low level of residual KatG activity is required for C10 to potentiate INH in the resistant isolates. In support of this model, when we generated a Δ*katG* strain that harbored a complete deletion of the *katG* locus, this mutant was completely resistant to INH even in the presence of C10, phenocopying the early stop mutant we had isolated (Figure 3.4).

Since some level of KatG activity was necessary for C10 to re-sensitize the resistant mutants to INH, we examined whether C10 impacted KatG activity both *in vitro* using purified KatG protein and *in vivo* (Figure 3.5 and Supplementary Figure 3.S4). Together these experiments showed that C10 did not directly or indirectly enhance KatG enzymatic activity. By LC-MS, we found that *katG* hypomorphic strains accumulate significantly decreased levels of active INH-NAD compared to WT (Figure 3.5). However, in all of these strains, C10 did not alter the level of INH-NAD in the cell. In contrast, the *katG*-null mutants that are resistant to INH even in the
presence of C10 were completely deficient in INH-NAD synthesis. Based on these findings, we concluded that C10 enhances the bactericidal effect of INH-NAD against \textit{Mtb} without increasing its accumulation, such that INH-resistant \textit{katG} hypomorphic strains that accumulate a sub-inhibitory level of INH-NAD become inhibited by this concentration of INH-NAD in the presence of C10. Consistent with this model, C10 was able to potentiate the bactericidal activity of the direct InhA inhibitor NITD-916 that is not known to be activated by KatG or any other activating enzyme (Figure 3.6) \cite{233}, demonstrating that the effect of C10 on INH sensitivity must occur downstream of INH activation. Therefore, C10 enhances the bactericidal effect of InhA inhibition.

This finding is notable because the overwhelming majority of small molecules that reverse bacterial drug resistance have been shown to do so by increasing the concentration of active antibiotic within the cell by inducing drug accumulation, blocking drug degradation, or increasing activation of pro-drugs \cite{130,132,134,136,147,148}. Furthermore, most known mechanisms of enhancing INH activity in WT bacteria involve increasing respiration or inhibiting DosR signaling to prevent bacteria from adopting a drug tolerant state \cite{19,119}, enhancing the accumulation of INH-NAD by modulating its activation or degradation \cite{125,126}, or titrating the expression or activity of the INH-NAD target InhA \cite{127}. We demonstrated that C10 does not work through any of these mechanisms. C10 inhibits respiration and does not inhibit DosR signaling (Figure 2.4 and Supplementary Figure 2.S4), and C10 does not impact INH-NAD levels during INH treatment (Figure 3.5). Additionally, by RNA-sequencing, C10 does not decrease the expression of \textit{inhA}, nor did C10 inhibit InhA activity in whole cell radiolabeling assays (Figure 3.6). Therefore, even though the mechanism by which C10 potentiates INH remains unknown, our data supports that it functions by a novel mechanism that has not yet been described.

\textbf{6.1.5 C10 sensitizes \textit{Mtb} to multiple clinically relevant antibiotics}
We examined whether C10 caused *Mtb* to become broadly more sensitive to any cell wall stress by monitoring bacterial survival in the presence of C10 with a panel of cell wall-targeting antibiotics. In addition to enhancing the bactericidal activity of InhA inhibitors, we found that C10 enhances the bactericidal activity of the peptidoglycan biosynthesis cocktail meropenem and clavulanate (Figure 3.7). However, C10 does not sensitize *Mtb* to all antibiotics non-specifically, since C10 significantly antagonized the bactericidal activity of arabinogalactan biosynthesis inhibitors and had no impact on the sensitivity to rifampicin (Figure 3.7). Therefore, C10 does not simply sensitize *Mtb* to any cell wall stress. Instead C10 makes *Mtb* specifically vulnerable to inhibitors of the mycolic acid or the peptidoglycan layers of the cell wall.

Additionally, we discovered that C10 enhances the bacterial sensitivity to killing by the respiration inhibitors Q203 and delamanid (Figure 2.6 and Figure 3.7), likely because of its impact on *Mtb* bioenergetics. We found that the ability of C10 to disrupt *Mtb* energy homeostasis is linked to its ability to inhibit *Mtb* growth at high concentrations (Figure 3.1 and Supplementary Figure 2.S5). Therefore C10 has two activities, at sub-inhibitory concentrations re-sensitizing some INH-resistant mutant to INH and causing potentiation of InhA inhibitors, meropenem/clavulanate, Q203, and delamanid against WT *Mtb*, and at higher concentrations inhibiting growth in a dose-dependent manner. Building on this growth-inhibitory activity, we developed more potent analogs of C10 with enhanced activity (Figure 4.1)(Anne Mayer-Bridwell and Souvik Sarkar, et al. unpublished), suggesting that future development on the 2-pyridone scaffold could yield more effective inhibitors of *Mtb* growth.

### 6.1.6 C10 limits *Mtb* pyruvate metabolism

In an attempt to understand the mechanism by which C10 inhibits *Mtb* energy homeostasis and growth, we discovered that C10 disrupts central carbon metabolism. Metabolite profiling of
C10-treated *Mtb* revealed that C10 causes depletion of bacterial pyruvate after 4 days of exposure (Figure 4.3). A C10-resistant mutant exhibited basal up-regulation of the glycerol assimilation pathway, ultimately resulting in a basal increase in pyruvate levels (Figure 4.2) and abrogating the ability of C10 to deplete bacterial pyruvate in this strain (Figure 4.3). Furthermore, supplementation with exogenous pyruvate and other metabolites that are linked to pyruvate metabolism rescued C10-mediated inhibition of *Mtb* (Figure 4.5 and Figure 4.6). Together, these findings converge on pyruvate limitation as the likely culprit responsible for the effects of C10 on *Mtb* energy homeostasis and growth, however it is still unclear how C10 leads to this effect.

It is possible that the enhanced INH sensitivity observed in the presence of C10 is due to depletion of bacterial pyruvate. However, C10 still potentiated killing by INH against the C10-resistant mutant in which C10 no longer depleted pyruvate levels (Figure 3.2). This finding suggests that depletion of pyruvate is likely not required for C10 to potentiate INH. Therefore, the mechanism by which C10 enhances INH remains un-resolved.

### 6.2 Open questions regarding C10

#### 6.2.1 What is the target (or targets) of C10?

The primary goal of the studies presented in Chapters 2-4 of this thesis was to understand the mechanism of action of C10. However, it still remains unknown what protein(s) C10 binds to within *Mtb* that elicit the effects that we observed. Identifying the bacterial target(s) of C10 would significantly help to elucidate its mechanism.

In future work, I would propose to identify C10-binding proteins using photoaffinity probes, wherein an analog of C10 is synthesized that has a UV-activatable crosslinking moiety as well as a tail that can be immunoprecipitated. We had poor success with early C10 photoaffinity
probes, as the analogs that we utilized pulled down a myriad of proteins non-specifically (data not shown). There is some additional troubleshooting that could be done to optimize the use of this probe. We could perform pull down experiments including high concentrations of C10 to compete with the probe for binding to real C10 targets, using proteomics mass spectrometry to quantify relative protein abundances across samples. This approach could allow us to differentiate between specific C10 binding partners, which should be decreased when C10 is included to out-compete the probe, compared to non-specific binding proteins, which should remain abundant even during competition with C10. Alternatively, the development of newer, more specific C10 probe analogs could aid in increasing the specificity of the probe for the target protein(s).

In a parallel approach, I would propose to perform cellular thermal shift assays (CETSA), in which either live *Mtb* or *Mtb* whole cell lysate is exposed to C10, and samples are incubated at increasing temperatures to denature proteins \(^293,294\). The denatured proteins precipitate from the solution and can be removed by filtration or centrifugation. Proteomics mass spectrometry is then used to quantify the soluble proteins across the temperature range. When bound to a ligand, proteins typically have an increased melting temperature compared to the protein alone. Therefore, I would expect that proteins that have increased thermal stability and remain soluble at higher temperatures in the presence of C10 could be putative binding partners of C10. Hits from both the photoaffinity and CETSA approaches would be confirmed in downstream biochemical assays, prioritizing proteins that were detected in both experiments.

Forward genetics is a powerful approach that is commonly used to understand the mechanism of action of small molecules. Through our forward genetic selection, we found that mutants that up-regulate *Rv0731c* or *lpdA-glplD2* or that harbor a single amino acid substitution in *Rv0830* have resistance to C10. It is possible that one or more of these genes encodes a target of
C10. To test this possibility, I would propose to purify each of these enzymes and perform in vitro ligand binding assays such as differential scanning fluorimetry or isothermal titration calorimetry. In a parallel approach, I would propose to genetically delete or deplete each of these enzymes to determine if genetic inhibition of these enzymes re-capitulates any of the effects of treating Mtb with C10. We have demonstrated that mutants up-regulating Rv0731c or lpdA-glpD2 are not resistant to the INH-potentiating effects of C10, so I would expect that if these genes do indeed encode a target of C10, this target is involved in the effects of C10 on energy metabolism, not on INH sensitivity.

There is some data to suggest that the enzymes encoded by the lpdA-glpD2 operon are not the physiologically relevant target of C10. We demonstrated that the strain up-regulating lpdA-glpD2 has increased flux through the glycerol assimilation pathway (Figure 4.2), suggesting that these enzymes participate in this process. If C10 inhibited glycerol assimilation by targeting LpdA, GlpD2, or another enzyme in this pathway, we would expect C10 treatment to cause accumulation of glycerol assimilation precursors such as glycerol-3-phosphate and deplete downstream products such as DHAP. In C10-treated Mtb, we see a modest increase in glycerol-3-phosphate, and a modest decrease in the abundance of DHAP, suggesting that C10 could decrease flux through this pathway in Mtb (Figure 4.3). Notably, there are 3 additional enzymes that are predicted to perform the function of GlpD2, so inhibition of one of these enzymes may not be sufficient to block this step of the glycerol assimilation pathway. However, to examine if the activity of C10 was dependent on glycerol metabolism, I tested whether C10 could inhibit Mtb growing in the absence of glycerol and found that C10 still inhibited Mtb during growth on glucose, pyruvate, or acetate as the primary carbon source (Figure 4.4). Therefore, while C10 might impact this pathway when Mtb is growing on glycerol, the activity of C10 is clearly not dependent on glycerol assimilation.
It remains possible that C10 binds LpdA or GlpD2, and their up-regulation causes C10 resistance by sequestering C10 and decreasing the concentration of free C10 in the bacteria, so it is still worthwhile investigating if C10 can bind these enzymes directly.

6.2.2 How does C10 impact Mtb metabolism?

Our preliminary metabolite profiling indicated that C10 can impact central carbon metabolism in Mtb, in particular leading to depletion of pyruvate levels, among other changes. Furthermore, we found that supplementing Mtb with the carbon sources propionate, pyruvate, and acetate rescued growth inhibition by C10, suggesting that access to these carbon sources can mitigate the toxicity of C10 (Figure 4.5 and Figure 4.6). Pyruvate is the final byproduct of glycolysis, and propionate can be converted to pyruvate through the methylcitrate cycle 296-299. Typically, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase, and acetyl-CoA can then feed into the first step of the TCA cycle, or a myriad of other pathways. Free acetate can readily be converted to acetyl-CoA by multiple enzymes 300. Therefore, the finding that propionate, pyruvate, and acetate can all rescue growth inhibition by C10 implicates the pyruvate-acetyl-CoA node of metabolism in the mechanism of action of C10. Since C10 treatment decreases α-ketoglutarate abundance in Mtb and leads to an increase in aspartate, which serves as a proxy for oxaloacetate within the cell, it is possible that C10 stalls the TCA cycle. However, supplementation with α-ketoglutarate or glutamate, which can readily be converted to α-ketoglutarate by a glutamate dehydrogenase, did not rescue the toxicity of C10, suggesting that simply rescuing the decrease in α-ketoglutarate is not sufficient to abrogate the toxicity of C10. In order to more precisely define the metabolic effects of C10 treatment, it is important to perform metabolic flux analysis, since compensatory pathways can alter the bulk pools of various metabolites, complicating the interpretation of these bulk metabolite analysis experiments. I would propose to
treat *Mtb* with C10 and include non-radioactive heavy $^{13}$C-labeled glycerol or acetate. By monitoring the flow of the $^{13}$C upon C10 treatment, we would be able to identify pathways that are impacted by C10 treatment and avoid the confounding effects of compensatory metabolic pathways. Our data is consistent with C10 disrupting *Mtb* ATP synthesis due to upstream effects on metabolism. However, without a more detailed carbon flux analyses it is difficult to determine which pathways are directly affected by C10 treatment. Using this flux analysis to pinpoint specific steps that are inhibited by C10, we would be able to identify metabolic enzymes that might be directly or indirectly affected by C10.

### 6.2.3 Does C10 inhibit menaquinone biosynthesis?

While our studies indicated that the effects of C10 on energy metabolism are likely separate from its effects on INH sensitivity, there is one compound in the literature that shares certain similarities with C10 in its ability to simultaneously inhibit *Mtb* respiration and potentiate killing by INH. The small molecule DG70 was identified in a screen for compounds that target the *Mtb* ETC $^{18}$. Selecting for DG70-resistant mutants resulted in the isolation of resistant strains harboring mutations in the menaquinone biosynthesis gene *menG* encoding the demethylmenaquinone methyltransferase required in one of the final steps of menaquinone biosynthesis $^{18}$. DG70 was shown to potently kill mycobacteria by inhibiting menaquinone biosynthesis, resulting in inhibition of *Mtb* respiration and depletion of bacterial ATP levels $^{18}$. Furthermore, DG70 was shown to enhance the antimicrobial activity of INH, rifampicin, and BDQ $^{18}$. This finding raises the possibility that inhibition of menaquinone biosynthesis in *Mtb* leads to simultaneous inhibition of ETC activity and enhanced INH bactericidal activity, unlike other respiration inhibitors $^{120-122}$.

This study raises the possibility that C10 similarly affects menaquinone biosynthesis, leading to inhibition of energy metabolism and increased INH sensitivity. One important
difference between C10 and DG70 is that C10 does not enhance the activity of rifampicin, whereas DG70 is synergistic with this antibiotic, suggesting that these compounds have different effects on Mtb antibiotic susceptibility and therefore C10 may not work through the same mechanism. However, to test this model, I would first confirm that inhibition of menaquinone biosynthesis potentiates killing by INH. I would propose to examine whether DG70 can still potentiate killing by INH in the menG mutant, to confirm that the potentiation of INH by DG70 is dependent on its ability to block menaquinone biosynthesis, and not due to off-target effects. In a complementary approach, I would genetically silence the expression of menG and other menaquinone biosynthesis genes using CRISPRi and expose the strains to INH to determine if decreasing menaquinone biosynthesis genetically can potentiate killing by INH. Alternatively, there are additional inhibitors of menaquinone biosynthesis that target other enzymes in the pathway. I would examine whether these other menaquinone biosynthesis inhibitors can potentiate killing by INH similar to DG70. If genetic or chemical inhibition of the menaquinone biosynthesis pathway potentiates the bactericidal activity of INH, this would support the model that DG70 enhances killing by INH through this mechanism and confirm that menaquinone biosynthesis is linked to INH sensitivity.

To test if C10 also impacts the menaquinone biosynthesis pathway, I would next propose to monitor the biosynthesis of menaquinone in C10-treated Mtb to determine if C10 inhibits MenG or another step in the biosynthetic pathway. I would measure the de novo synthesis of menaquinone by 14C-labeling live Mtb with the radioactive methyl-donor 14C-methionine and performing thin layer chromatography to determine if C10 blocks menaquinone biosynthesis. Additionally, total pools of menaquinone and its precursors can be extracted from C10-treated Mtb and measured using mass spectrometry to determine if C10 disrupts menaquinone biosynthesis. If C10 is also shown to inhibit the menaquinone biosynthesis pathway, similar to DG70, then this would provide
a missing link between the effects of C10 on energy metabolism and INH sensitivity.

**6.2.4 How does C10 sensitize *Mtb* to InhA inhibition?**

Regardless of how C10 impacts central carbon metabolism and/or menaquinone biosynthesis, the question remains how C10 specifically sensitizes *Mtb* to killing by the InhA inhibitors INH and NITD-916. In our $^{14}$C-labeling experiment to monitor *de novo* mycolic acid biosynthesis as a metric for InhA activity, we found that C10 did not decrease mycolic acid biosynthesis on its own or in combination with INH (Figure 3.6). A major limitation of this experiment is the short 20 hour incubation time. It is possible that treating *Mtb* with C10 and/or INH and labeling with $^{14}$C-acetate for longer periods of time might resolve differences in mycolic acid biosynthesis that only occur after longer exposure to these compounds. It is therefore still possible that C10 enhances the ability of INH-NAD to inhibit InhA, which would be supported if C10 enhances the inhibition of mycolic acid biosynthesis by INH treatment in WT or *katG*<sup>W328L</sup> *Mtb* at later time points. Additionally, to test if C10 directly promotes the inhibition of InhA by INH-NAD, we could purify InhA protein and perform *in vitro* enoyl-ACP dehydrogenase activity assays in the presence of C10 and/or INH-NAD or NITD-916<sup>113,233</sup>. These experiments would allow us to determine if C10 enhances the ability of INH-NAD or NITD-916 to inhibit InhA.

Alternatively, it is possible that C10 impacts a pathway independent of InhA in *Mtb* that makes the bacteria more vulnerable to the inhibition of mycolic acid biosynthesis. For example, C10 might inhibit the synthesis of a cell wall component that can partially compensate for the loss of integrity in the mycolic acid layer during INH or NITD-916 treatment. There are a myriad of cell wall lipids and glycolipids that are present in the *Mtb* cell envelope in addition to the mycolyl-arabinogalactan-peptidoglycan complex. While some of these molecules have a known function, the roles of many of these lipids remains unknown. One piece of evidence that supports the
possibility that C10 inhibits the synthesis of a cell wall component is the presence of an unidentified band on the TLC plates in our $^{14}$C-acetate labeling experiment that is absent in samples containing C10 (Figure 3.6 and Supplementary Figure 3.S7). This band appears directly above the band predicted to be TMM, and therefore might also be a cell wall glycolipid. Perhaps by inhibiting the synthesis of this product, C10 makes the *Mtb* cell wall particularly vulnerable to disruption of the mycolic acid layer during INH or NITD-916 treatment. To investigate this model, I would propose to perform TLC on non-radioactive *Mtb* extracts prepared similarly to the samples in our experiments, and to use tandem mass spectrometry in combination with other physical chemistry approaches to try to identify the structure of the unknown molecule in this TLC band. In a parallel approach, I would treat *Mtb* with C10, purify the insoluble cell wall, and quantify the cell wall sugars by acid hydrolysis and liquid chromatography to identify differences in the sugar composition of C10-treated *Mtb*, which could point to whether C10 impacts the synthesis of specific glycan-containing cell wall components.

6.2.5 Clinical development of C10

Ultimately, our long-term goal is to identify small molecule clinical candidates that could be used to enhance the current antimicrobial regimen in the clinic. To determine if C10 or its related analogs hold promise for downstream clinical development, I would propose to perform mouse experiments to measure the *in vivo* efficacy of these compounds. As C10 inhibits *Mtb* energy metabolism, which is a bona fide drug target of existing anti-TB antibiotics $^{156,239,303}$, I would first test if C10 has the capacity to inhibit *Mtb* growth in mice on its own. Since the IC$_{50}$ of C10 is approximately 25μM *in vitro*, I anticipate that it would be difficult to treat mice with enough C10 to reach inhibitory concentrations. I would instead propose to use more potent C10 analogs such as PD217 and SS451. I would examine whether these analogs can inhibit the growth of *Mtb*.
in mice, either during the acute phase of infection, or in an established infection model.

Additionally, the feature of C10 that is most attractive for clinical development is its synergistic killing in the presence of other antibiotics. In particular, the ability of C10 to enhance the susceptibility of both WT and katG mutant Mtb to inhibition by INH indicates that C10 could be used to re-sensitize INH-resistant strains to this important antibiotic. I would test for the ability of C10, PD217, or SS451 to enhance the bactericidal activity of INH in mice using both WT and katG mutant strains of Mtb. Since the concentration of C10 required is quite high (~5-25μM), I anticipate that it would be difficult to reach an effective concentration of C10 in mice. Therefore, the more potent analogs PD217 or SS451 may prove more effective. Additionally, I would propose to examine whether these 2-pyridones potentiate Q203, delamanid, or meropenem/clavulanate in mice, to determine if C10 could be used as an adjuvant therapy to enhance the efficacy of these other antibiotics.

It is possible that the physicochemical properties of C10 and other bicyclic 2-pyridones are not optimal for use in vivo. Therefore, once the mechanism of action of C10 is better understood, I would propose to use a genetic approach to delete or conditionally silence the expression of the C10 target(s), once they are identified, to determine if genetic inhibition of these targets leads to either decreased growth or to enhanced killing by INH in mice. After genetically confirming that the target(s) of C10 are important during growth in mice or for survival during INH treatment, I would propose to develop a target-based small molecule screen to increase the chemical diversity of scaffolds that inhibit the same target(s) as C10. By identifying structurally diverse compounds that inhibit Mtb through a similar mechanism, we could increase the chances of identifying a compound that has more favorable chemical properties for clinical development.

6.3 Discovery of a novel QcrB inhibitor
In an approach to screen for new compounds with anti-mycobacterial activity, we identified a class of nucleotide mimetics called 4-amino-thienopyrimidines, which exhibit bacteriostatic activity against *Mtb*. We worked with chemistry collaborators to generate a library of 4-amino-thienopyrimidines and performed structure-activity-relationship studies to generate analogs with potency in the nanomolar range (Supplementary Table 5.S1 and Supplementary Table 5.S2). In order to understand how these compounds inhibit *Mtb*, we took a forward genetic approach to isolate mutants that were resistant to these compounds. Each mutant strain that we sequenced harbored a mutation in the gene *qcrB*, which encodes a subunit of the cytochrome *bc1:aa3* oxidase in the ETC that is a validated drug target against *Mtb* (Table 5.1)\(^{156-161,274,282}\). When we mapped these mutations onto a predicted model of the QcrB crystal structure, we found that all the amino acid substitutions were located in or near the predicted menaquinol binding site of the enzyme (Figure 5.5). Furthermore, using molecular docking approaches, the 4-amino-thineopyrimidine compound CWHM-728 was predicted to bind within this active site (Figure 5.5). By genetically manipulating the parental strain to introduce one of these mutations, an A172T substitution, we demonstrated that this single amino acid change is sufficient to confer resistance to 4-amino-thienopyrimidines (Figure 5.4). Based on these findings, we hypothesized that this series of compounds targets QcrB. We tested if 4-amino-thienopyrimidines could inhibit the ETC by treating *Mtb* or *M. smegmatis* with the compounds and measuring bacterial ATP. We found that, consistent with inhibition of the ETC, 4-amino-thienopyrimidines depleted bacterial ATP (Figure 5.6). Furthermore, we demonstrated that a ΔcydA mutant lacking the compensatory cytochrome *bd* oxidase was significantly more sensitive to inhibition by 4-amino-thienopyrimidines, which is a hallmark of QcrB inhibitors (Figure 5.7)\(^{157,160,166}\). Collectively, these findings support our model that these compounds target QcrB.
6.4 Open questions regarding 4-amino-thienopyrimidines

Our finding that 4-amino-thienopyrimidines have enhanced potency against a ΔcydA mutant highlights the potential utility of developing combination therapy that simultaneously targets both cytochrome \( b_{c1:aa3} \) and cytochrome \( bd \) oxidases. Indeed, it has been demonstrated that deletion of cytochrome \( bd \) can cause QcrB inhibitors, which are typically bacteriostatic, to become bactericidal \(^{160,166}\). Based on this concept, there has been some work from other labs to develop cytochrome \( bd \) oxidase inhibitors \(^{169-172}\). It is likely that simultaneously treating patients with a cytochrome \( bd \) inhibitor would significantly improve the anti-mycobacterial activity of a cytochrome \( b_{c1:aa3} \) inhibitor in the clinic.

Intriguingly, a recent study published after our initial description of 4-amino-thienopyrimidines as anti-mycobacterial compounds demonstrated that altering the substituents on the same 4-amino-thienopyrimidine backbone could actually produce a compound that inhibits cytochrome \( bd \) oxidase \(^{171}\). This finding suggests that the 4-amino-thienopyrimidine scaffold can be modified to target either cytochrome \( b_{c1:aa3} \) oxidase or cytochrome \( bd \) oxidase, and raises the interesting theoretical prospect of developing a single compound that could target both oxidases simultaneously. We found that the 4-amino-thienopyrimidine CWHM-1023 caused \(~50\%\) inhibition against the \( \Delta qcrCAB \) \( M. \text{smegmatis} \) mutant (Figure 5.4), which we concluded is due to a small amount of off-target activity of CWHM-1023 against another target in \( M. \text{smegmatis} \). Due to its structural similarity to the recently developed cytochrome \( bd \) inhibitors \(^{171}\), it is possible that CHWM-1023 is actually a weak inhibitor of the \( M. \text{smegmatis} \) cytochrome \( bd \), causing approximately 50\% growth inhibition when cytochrome \( b_{c1:aa3} \) is missing.

Since both oxidases have a menaquinol binding site, and therefore bind the same substrate,
it could be feasible to design an inhibitor that binds the active site of both enzymes. This undertaking would require renewed medicinal chemistry efforts as well as the development of screening methods that can confirm the biological activity of these compounds. Developing in vitro assays of cytochrome \( b_{c1}:a_{a3} \) or cytochrome \( b_d \) activity using purified protein complexes would aid in our ability to confirm whether compounds have bifunctional inhibitory activity. The structures of both the mycobacterial cytochrome \( b_{c1}:a_{a3} \) oxidase and the cytochrome \( b_d \) oxidase have been resolved by cryo-electron microscopy\(^{304-306} \), which could facilitate the rational design of chemical modifications that improve binding of 4-amino-thienopyrimidines to the active sites of both enzymes. Against live \textit{Mtb}, we predict that compounds that simultaneously inhibit both complexes should have bactericidal activity rather than the bacteriostatic activity that is characteristic of cytochrome \( b_{c1}:a_{a3} \) inhibitors.

Additionally, we can use the tools that we have already developed to devise a screen for novel cytochrome \( b_d \) inhibitors. By screening for compounds that selectively inhibit the \( \Delta qcrCAB \) \textit{M. smegmatis} mutant, and not WT \textit{M. smegmatis}, we could identify small molecules that might enhance the antibacterial activity of cytochrome \( b_{c1}:a_{a3} \) inhibitors. Since cytochrome \( b_d \) is non-essential when the bacteria have an intact cytochrome \( b_{c1}:a_{a3} \) oxidase, specific inhibitors of cytochrome \( b_d \) should have no inhibitory activity against WT \textit{M. smegmatis}. In theory, in addition to identifying compounds that enhance cytochrome \( b_{c1}:a_{a3} \) inhibitors through inhibition of cytochrome \( b_d \), this approach has the potential to identify compounds that would enhance cytochrome \( b_{c1}:a_{a3} \) inhibitors through any mechanism. In order to improve our ability to screen for compounds that work against \textit{Mtb} we could replace the \textit{M. smegmatis} cytochrome \( b_d \) encoding genes (\textit{cydAB}_{\textit{M. smegmatis}}) with the \textit{Mtb} orthologs (\textit{cydAB}_{\textit{Mtb}}), which would help to specifically identify inhibitors of the \textit{Mtb} enzyme. Ultimately, lead compounds identified through this
approach can be confirmed for activity against *Mtb*, to determine if the newly identified compounds potentiate the bactericidal activity of known cytochrome *bc1:aa3* inhibitors.

### 6.5 Final Remarks

By investigating the mechanism of action of small molecules with activity against *Mtb*, this work highlights the possibility of circumventing *Mtb* drug resistance by identifying small molecules that reverse resistance or inhibit novel antimycobacterial targets. I characterized one compound, C10, which has the exciting potential to disarm isoniazid resistance through an unknown mechanism. In an attempt to uncover the mechanism of action of C10, I systematically ruled out a number of models. However, through this process, I identified multiple clinically relevant antibiotics that are potentiated by this compound and challenged paradigms in the field about the link between energy metabolism and INH activity. Still, the mechanism of action of C10 remains enigmatic. By characterizing the effects of C10 on *Mtb* energy metabolism and identifying a new chemical scaffold that inhibits the validated drug target QcrB, this work emphasizes that metabolism and respiration are druggable pathways that can be exploited for the development of new antimycobacterial antibiotics.
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