Genetic and chemical validation of dihydrofolate reductase as a potential target for the treatment of NTM infections

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Nowrin Hoque wrote the introduction and literature review. Marcel A. Behr edited the draft and authorized submission of the final version of the chapter.

Chapter II. ***Validation of dihydrofolate reductase as a therapeutic target for the treatment of M. abscessus infection*** **(manuscript).**

Marcel A. Behr conceived the study. Nowrin Hoque and Marcel A. Behr set up the experimental design for the manuscript. Design and preparation of CRISPRi plasmids was performed by Nowrin Hoque (for *M. smegmatis* and *M. abscessus*) and Marwan Ghanem (for *M. smegmatis*). Nowrin Hoque performed electroporation, RNA extraction and confirmation of gene knockdown by qRT-PCR. Chris Walpole (from the SDDC) and David Sherman provided the DHFR inhibitors. Nowrin Hoque performed dose-response experiments of the SDDC compounds by broth and agar dilution. Nowrin Hoque and Jaryd Sullivan performed inter-assay variability experiments of AMK, RIF and BDQ. Generated experimental data was analyzed by Nowrin Hoque and Marcel Behr.

Chapter III. Reflections and future directions.

Nowrin Hoque wrote the reflections and future directions. Marcel A. Behr edited the draft and authorized the final version of the chapter.

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LIST OF ABBREVIATIONS

ADC – Albumin dextrose catalase
AMK – Amikacin
ATC – Anhydrotetracycline
ATCC – American type culture collection
ATP – Adenosine triphosphate
BDQ – Bedaquiline
BRET – Bioluminescent Resonance Energy Transfer
CCCP – Carbonyl cyanide 3-chlorophenylhydrazone
cDNA – Coding DNA
CF – Cystic fibrosis
CFTR – Cystic fibrosis transmembrane conductance regulator
CFU – Colony-forming unit
CLSI – Clinical and Laboratory Standards Institute
COPD – Chronic obstructive pulmonary disease
CRISPRi – Clustered regularly interspaced short palindromic repeat interference
DST – Drug susceptibility testing
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
DosR – Dormancy survival regulator
dTMP – Deoxythymidine monophosphate
dUMP – Deoxyuridine monophosphate
dUTP – Deoxyuridine triphosphate
HIV – Human immunodeficiency virus
INH – Isoniazid
MAC – Mycobacterium avium Complex
MAB – *Mycobacterium abscessus*
MIC – Minimum inhibitory concentration
MmpL – Mycobacterial membrane protein large
MmpS – Mycobacterial membrane protein small
MTB – *Mycobacterium tuberculosis*
MTBC – MTB Complex
NADPH – Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NTM – Nontuberculous mycobacteria
NTM-PD – NTM pulmonary disease
OD_{600} – Optical density at 600 nm
PAM – Protospacer adjacent motif
PAS – *para*-aminosalicyclic acid
PCR – Polymerase chain reaction
qRT-PCR – Quantitative reverse transcriptase-PCR
REMA – Resazurin Microtiter Assay
RIF – Rifampicin
RNA – Ribonucleic acid
rRNA – Ribosomal RNA
RGM – Fast-growing mycobacteria
SDDC – Structure-guided Drug Discovery Coalition

SGM – Slow-growing mycobacteria
sgRNA – Single guide RNA
TB – Tuberculosis
TNF-α – Tumor necrosis factor alpha
WGS – Whole-genome sequencing

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ABSTRACT

Nontuberculous mycobacteria (NTM) are emerging opportunistic pathogens that can cause chronic, tuberculosis (TB)-like lung disease. One of the most clinically relevant and drug resistant NTM species is *Mycobacterium abscessus* (MAB). The success rate of treatment with antibiotics is low, due to the fact that NTM are intrinsically resistant to many of the standard anti-TB drugs. Patients undergo a prolonged multi-drug regimen, where treatment-associated toxicity is considerable. Despite the poor outcomes of therapy, there is limited NTM drug discovery work. Current efforts are limited by the lack of extensive knowledge on NTM drug targets and challenges in NTM drug susceptibility testing (DST) in the laboratory. This study aims to discover new drugs against MAB, where the guiding hypothesis is that anti-TB drug targets offer the most direct path to new antibiotics for NTM, but that we need new chemistry to act at these established targets. We used a combination of CRISPRi and chemistry to test whether dihydrofolate reductase (DHFR) would be a valid target for the treatment of MAB. We also set out to examine inter-assay variability when performing DST for known anti-TB drugs.

In preliminary work, the CRISPRi methodology has been first validated in the model organism *M. smegmatis* and subsequently in MAB. With this technique, the DHFR gene, *dfrA*, was observed to be essential for *in vitro* growth of two subspecies of MAB. A series of candidate DHFR inhibitors has been tested for *in vitro* growth inhibition using broth microdilution and conventional agar dilution method. A number of these compounds showed potent antimicrobial activity against MAB (MIC90 at 10μM or lower). In the current study, inter-assay variability was assessed when measuring MIC of amikacin (AMK), rifampicin (RIF) and bedaquiline (BDQ) in MAB, using the following assays: agar dilution, OD_{600}, BacTiter-Glo and AlamarBlue. Highest reproducibility was achieved by agar dilution and OD_{600} while AlamarBlue, and particularly BacTiter-Glo, gave inconsistent results.

Together, these data suggest that DHFR can serve as a promising drug target for NTM infections, as shown via CRISPRi and chemical inhibition for MAB. We also found that different assays used for DST may yield discrepant results depending on the drug. Our data suggest that the OD_{600} assay is most suitable to guide compound selection for drug discovery against NTM. The lack of successful treatment continues to compromise health outcomes of patients. Novel therapeutic compounds are urgently needed for the treatment of NTM infections. This study aims to address this unmet need.

RÉSUMÉ

Les mycobactéries non tuberculeuses (MNT) sont des agents pathogènes opportunistes émergents responsables de maladies pulmonaires graves et chroniques, semblable à la tuberculose (TB). Une des espèces de MNT la plus cliniquement pertinente est le *Mycobacterium abscessus* (MAB), démontrant une très haute résistance aux antibiotiques. Le taux de succès des traitements est faible dû à leur résistance intrinsèque à de nombreux médicaments antituberculeux. Les régimes thérapeutiques actuels sont longs et la toxicité associée au traitement est considérable. Malgré les résultats médiocres de la thérapie, la découverte de médicaments contre les MNT est limitée. Les efforts actuels sont limités par le manque de connaissances approfondies sur les cibles médicamenteuses des MNT et par les difficultés rencontrées lors de l'effectuation des tests de sensibilité en laboratoire. Cette étude vise à découvrir de nouveaux médicaments contre le MAB. Notre hypothèse est que de nouveaux composés contre les cibles des médicaments antituberculeux actuels offrent la voie la plus directe pour la découverte de nouvelles thérapies antimicrobiennes contre les MNT. Nous avons utilisé une combinaison de CRISPRi et de chimie pour vérifier si la dihydrofolate réductase (DHFR) constituerait une cible valable pour le traitement du MAB. Nous avons également examiné la variabilité entre différents essais de sensibilité pour des médicaments antituberculeux connus.

La méthodologie de CRISPRi a d'abord été validée dans l'organisme modèle *M. smegmatis*, puis dans le MAB. Avec cette technique, le gène DHFR, dfrA, s'est révélé essentiel pour la croissance *in vitro* de deux sous-espèces de MAB. Une série d'inhibiteurs de DHFR candidats a été testée pour l'inhibition de croissance *in vitro* en utilisant la microdilution en milieu liquide et la méthode de dilution en gélose conventionnelle. Quelques composés ont montré une activité antimicrobienne significante contre le MAB (CMI 90 à 10 μM ou moins). Dans la présente étude, la variabilité inter-essais a été évaluée lors de la mesure de la CMI de l'amikacine (AMK), de la rifampicine (RIF) et de la bédaquiline (BDQ) dans le MAB, en utilisant les tests suivants: dilution en gélose, OD600, BacTiter-Glo et AlamarBlue. La reproductibilité été la plus élevée pour la dilution en gélose et l'OD600, tandis qu'AlamarBlue, et en particulier BacTiter-Glo, donnait des résultats incohérents.

Ensemble, ces données suggèrent que la DHFR peut constituer une cible médicamenteuse prometteuse pour les infections à MNT, comme indiqué par CRISPRi et l'inhibition chimique du

MAB. Nous avons également constaté que différents essais de sensibilité pouvaient donner des résultats discordants dépendamment du médicament. Nos données suggèrent que le test OD600 est le plus approprié pour guider la sélection de composés en vue de la découverte de médicaments contre MNT. L'absence de traitement efficace continue de compromettre la santé des patients. De nouveaux composés thérapeutiques sont nécessaires contre les infections à MNT. Cette étude cible à combler ce besoin critique afin d'améliorer la qualité de vie des patients affectés.

CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

1. Mycobacteria

1.1. Taxonomy

The *Mycobacterium* genus, part of the *Actinobacteria* phylum, consists of rod-shaped, non-motile, aerobic bacteria[1]. They are famously known for their thick, lipid-rich, double-layered cell wall, appearing acid-fast when stained with Ziehl-Neelsen stain. Species are classified according to growth rate: slow-growing mycobacteria (SGM) (take 7 days or more to grow) or fast-growing mycobacteria (RGM) (grow within 7 days)[2]. The majority of mycobacterial species are known as nontuberculous mycobacteria, which are considered separate from the *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium leprae* due to their inability to cause tuberculosis or leprosy. Further classification of NTM is according to pigment formation: SGM consist of Runyon group I, II and III, while RGM comprise of Group IV.

Runyon group I: photochromogens, i.e. forms pigment upon light exposure
(e.g. *Mycobacterium kansasii* and *Mycobacterium marinum*)
Runyon group II: scotochromogens, i.e. forms pigment in the dark or light
(e.g. *Mycobacterium gordonae* and *Mycobacterium scrofulaceum*)
Runyon group III: non-photochromogens, i.e. non-pigmented
(e.g. *Mycobacterium avium* and *Mycobacterium intracellulare*)
Runyon group IV: do not produce pigment
(e.g. *Mycobacterium abscessus* (MAB) and *Mycobacterium smegmatis*)

In addition to obligate pathogens (e.g. *M. tuberculosis* (MTB) and *M. leprae*), mycobacteria largely comprise of strictly commensal or saprophytic species (e.g. *M. smegmatis*) as well as several opportunistic pathogens (e.g. *M. avium*, MAB and *M. kansasii*)[3]. The use of 16S rRNA gene sequencing and whole-genome sequencing (WGS) has greatly facilitated the characterization of phylogenetic relationships, as well as the tracking of NTM transmission[4]. Genome comparison studies show that NTM are enriched in species-specific genes particularly

with regards to their energy metabolism, and have larger genomes compared to mycobacterial obligate pathogens [3]. The larger genome consists in part of genetic redundancy where functional gene duplicates allow NTM to achieve robustness when facing uncertain environments[3, 5]. On the other hand, MTB underwent extensive genome reduction upon adapting to the predictive environment of its intracellular host, where such level of robustness is arguably not needed.

2. Nontuberculous mycobacteria (NTM)

2.1. History and epidemiology

To date, over 170 NTM species naturally reside amongst soil and aquatic environments, distinguishing them from the strictly human pathogen MTB[3]. Within the host or environmental reservoir, NTM can survive as free-living organisms, in amoebas or exist in a biofilm-associated state. Historically, all NTM were considered saprophytic and it was only until the 1950s that their potential to cause disease in humans and in certain animals was recognized[6]. A number of NTM species are airborne opportunistic pathogens while others can cause disease in immunocompetent individuals. Pulmonary infection by RGM is most commonly caused by MAB subspecies; for SGM it is most commonly caused by *Mycobacterium avium* complex (MAC: *M. avium* and *M. intracellulare*), *M. kansasii* and *M. xenopi*. Nonpathogenic NTM can colonize human skin, respiratory, gastrointestinal and genitourinary tracts[3]. Distribution of NTM varies geographically but in many areas the most prevalent clinically relevant NTM species are MAC, MAB and *M. kansasii*[7, 8].

The incidence and prevalence of NTM disease has been on the rise worldwide and has surpassed TB incidence in many industrialized countries including Canada and the U.S.[2, 9, 10]. Pulmonary disease incidence is increasing in both the immunocompromised and immunocompetent populations, where the latter largely comprises of nosocomial infections[3]. In Ontario, from 1998-2010, the prevalence of pulmonary NTM disease rose from 29.3 cases/100,000 persons to 41.3/100,000[10]. In the U.S. population, the average age-adjusted prevalence increased from 8.7 to 13.9 per 100,000 persons from 2008 to 2013[11]. While improved culturing technique has facilitated the diagnosis of NTM infection and differentiation

from TB cases, it does not entirely account for the surge in disease prevalence. A true increase in disease prevalence, and a greater awareness of NTM's potential to cause disease, have also been attributed to this phenomenon. In addition to higher NTM isolation rates, more patients are meeting clinical criteria for active infection[2].This is likely due to a growing prevalence of patients at risk: aging population, higher number of organ transplants and co-morbidity with other infections. A heightened use of immunosuppressants such as corticosteroids and TNF-α inhibitors may also explain the rise in prevalence. Increased referral to a specialist and heavier surveillance for NTM through frequent testing may also explain the higher rate of isolation.

It remains challenging to accurately estimate NTM disease prevalence for various reasons. For TB, each clinical isolate is assumed to represent a case of TB disease; this is not the case for NTM[8]. Culturing NTM from a clinical sample does not necessarily mean that a patient has an active infection. Furthermore, public health reporting is not mandatory, unlike with TB. Additionally, currently available epidemiological data for NTM is not representative of worldwide prevalence. Data exist for only a subset of geographic regions including United States, Japan and Europe. Finally, misdiagnosis of NTM cases for TB is common in resource-limited settings due to shared clinical manifestations and similar appearance under microscopic examination[12]. This leads to an underestimate of incidence while also posing as a barrier to effectively eliminate TB due to wasting of resources[13].

The type of NTM infection depends on host risk factors, the source of infection and the microorganism itself. Approximately 65-90% of NTM cases are respiratory infections where patients with impaired airway clearance mechanisms and pre-existing lung damage are most vulnerable[2]. These include patients with cystic fibrosis (CF), chronic obstructive pulmonary disorder, bronchiectasis or patients with a history of TB. The risk of pulmonary NTM infection was found to be 29-fold higher in COPD patients receiving inhaled corticosteroid therapy[14]. These patients have chronic inflammatory infections with clinical manifestations similar to TB. In addition to progressive decline in lung function, symptoms may include sputum production, chronic cough, night sweats, weight loss, fever and chest pain[15]. Co-morbidity between NTM and TB in patients undergoing anti-TB therapy does occur; 7.1% of patients according to one

study[16].

Disseminated disease may also occur, exclusively in patients exhibiting a systemic immune deficiency such as HIV, where NTM can be cultured from the blood[8]. Co-morbidity between NTM and HIV is common in Southern African countries. Increased use of anti-retroviral therapy and prophylactic use of macrolides have reduced the incidence of disseminated NTM[17]. Other forms of NTM disease include extrapulmonary, skin and soft tissues and lymphadenitis. Immunocompromised individuals or patients receiving immunosuppressants are predisposed to such infections[18].

It should be noted that immunocompetent people can also get infected by certain NTM species. In fact, in many cases of NTM infection in children the presence of risk factors appears to be unknown where such patients develop cervical lymphadenitis[8]. Young healthy children are susceptible to *M. kansasii* infection. Disease prevalence is more prominent in women than in men and it increases with age[19, 20].

2.2. Method of infection

NTM infections can be acquired by inhalation or ingestion of soil aerosols, contaminated water, or inoculation on exposed skin surface[15]. These organisms can be found in swimming pools, hot tubs, treated drinking water dispensers, household plumbing, drainage water and hospital water systems[21]. Interhuman transmission is typically not reported for NTM infections. However, recently there has been evidence of indirect human-to-human transmission in CF patients through cough aerosols[4]. While colonization and infection of NTM in livestock and wildlife can occur, there is no evidence of transmission between humans and animals[15].

2.3. Pathophysiology

Most NTM infections are pulmonary where the bacterial agent enters the respiratory tract and penetrates into the mucosa, upon which alveolar macrophage-led phagocytosis of the bacteria occurs. Typically, under optimal conditions, a bacteria-containing phagosome is formed in which reactive oxygen species are released and acidification occurs[22]. This is followed by induction of autophagy and apoptosis leading to bacterial cell death. However, NTM have developed strategies

to withstand these killing mechanisms and persist in the host. In immunocompromised patients, a poorly formed granuloma or insufficient elicitation of an inflammatory response occurs[23]. In immunocompetent patients, the histology depends on the patient where necrotising or non-necrotising granulomas with inflammation are possible.

Intra-phagosomal survival is achieved by MAB by restricting acidification levels, inducing less apoptosis and by blocking autophagy flux[24]. Extracellular replication can also occur. Similar to MTB, upon nitric oxide-induced hypoxia, the dormancy survival regulator (DosR) regulon is strongly upregulated and is hypothesized to be essential for MAB persistence within the host during prolonged treatment[25]. In *M. avium*, a state of dormancy has already been demonstrated in response to nutrient starvation[26]. It may be that NTM is capable of entering a nonreplicating state, enabling drug resistance.

NTM species are heterogenous with respect to their virulence and ability to cause disease, which may also vary at the inter-strain level. An important mechanism of NTM pathogenesis is the formation of biofilm (e.g. on catheters and other medical equipment)[21, 27]. This confers protection from environmental threats such as exposure to antibiotics or disinfectants. A noteworthy distinction from MTB is NTM's ability to achieve different colony morphology, switching from antibiotic-susceptible to resistant variant. Isolates of MAC can take the form of either opaque or transparent (virulent) colonies, while MAB can exhibit smooth or rough (virulent) morphology[27, 28].

2.4. Diagnosis and treatment

Due to the ubiquitous nature of NTM in the environment, isolation does not equate infection. A culture-positive sputum sample is not sufficient to begin antimicrobial therapy, where additional clinical and radiological evidence is required[8]. Isolation of NTM from a clinical specimen may be due to contamination from the environment from using unsterile water or contaminated healthcare equipment. Respiratory samples that were culture-positive met clinical criteria for active infection in only 50% of the cases[29]. In such instances, additional sputum samples must be collected and examined.

Moreover, the decision to start NTM therapy depends on the nature of the isolate, the severity of the disease, and the clinician's judgment. The cure rate for NTM pulmonary disease is discouraging; it ranges from 30-50% for MAB [30]. An individualized analysis of risks and benefits to start therapy is required. Clinicians may choose to not treat the infection due to poor treatment outcome, treatment-associated toxic side effects and suboptimal adherence rate of patients[31]. Recurrent infections can occur, either relapse of infection caused by the original NTM strain or reinfection with a new strain. Recurring infections are due to the wide array of antibiotic resistance mechanisms in NTM that will be later discussed in this paper in more dept. Besides drug resistance, a notable barrier to optimal patient outcomes is that currently available clinical guidelines often group together treatment options for different species and subspecies despite variable drug susceptibility profiles. Comorbidity of multiple species of NTM in an infection is not uncommon, and this further complicates treatment options[32].

The course of treatment is long, typically between 6-24 months, where antibiotics for MAC infection are prescribed for 12 months following sputum conversion[15]. Patients undergo multidrug-regimens, often consisting of a minimum of three antibiotics, with severe side effects that can lead to hearing loss, hepatoxicity, neurological and gastrointestinal disease. For pulmonary MAC, a drug combination of macrolide, rifampicin and ethambutol is typically prescribed. Treatment of MAB pulmonary disease is typically 6 months and consists of a macrolide, aminoglycoside and either cefoxitin, imipenem or tigecyline. In case of drug therapy failure, occurrence of hemoptysis or formation of an abscess, surgery may be performed.

2.5. Drug resistance

Intrinsic and acquired drug resistance represent the main drivers of poor treatment outcome in NTM infections. These microorganisms have already adapted to survive within the rigorous conditions of their environment, including a diversity of antimicrobial compounds released by competitor environmental bacterial species. Poor cell permeability of the drug and its inability to accumulate intracellularly due to efflux pumps are a major mechanisms of drug resistance in NTM (e.g. fluoroquinolone and macrolides)[33]. The thick hydrophobic, bilayer cell membrane of NTM constitutes 30% of the total cell mass[34]. It enables resistance to high temperatures, low pH, hydrophilic agents, disinfectants and antibiotics. Moreover, reversible colony morphology switch

has been reported for *M. avium* and MAB that changes the cell wall permeability to drugs, this has been referred to as morphotypic antibiotic resistance[27, 35].

Expression of NTM enzymes to modify the drug target or the antibiotic itself are other mechanisms of intrinsic resistance [36]. NTM may metabolize an antibiotic into a less active form through expression of mycobacterial enzymes that can modify the drug (e.g. beta-lactamase and acetyltransferases). Due to genetic redundancy, certain drug targets suitable against other pathogenic bacteria may not be for NTM[13]. This may explain why many of the current broad-spectrum antibiotics are ineffective against these organisms. Horizontal gene transfer of resistance-conferring genes in NTM has been reported[37]. Moreover, NTM possess the transcription factor WhiB7, conserved across other mycobacterial species, that induces the expression of many genes essential for intrinsic drug resistance including clarithromycin, amikacin, erythromycin and tetracycline[38].

Acquired resistance in NTM has been reported, however mutation frequency and mechanisms remain elusive[13]. Prolonged treatment promotes emergence of acquired resistant mutations that become inherited by the offspring, leading to a population of resistant bacteria. Mutations at the docking site of a target gene can affect the drug's binding affinity. Alternatively, genetic mutations in other related genes may arise that blocks the drug's access to the active site of the targeted protein.

3. *Mycobacterium abscessus*

3.1. History and epidemiology

In 1952, the first isolate of MAB was obtained from a knee abscess in a 63-year-old woman and was named as such due to its ability to cause subcutaneous abscesses[39]. Its virulence was originally underestimated as it was thought to only be able to produce self-limiting skin infections[40]. Four decades later, its capacity to cause pulmonary disease was recognized. Prior to improved culturing techniques and genetic sequencing, MAB was originally undistinguishable from *M. chelonae*. As a result, many studies have mistakenly reported findings for one species despite culturing for both. To date, three subspecies of MAB have been identified: *M. abscessus abscessus, M. abscessus massiliense, M. abscessus bolletti*. Published clinical guidelines for CF

patients recommend similar antibiotic regimens for these subspecies, however differences in drug susceptibility pattern across subspecies have been described [41]. This becomes a barrier to proper NTM disease management.

3.2. Infection in Cystic Fibrosis population

Patients with impaired immune airway defense mechanisms are most vulnerable to MAB disease. Patients with CF represent the leading population at risk of such infections (worldwide prevalence rate of 3-20%) where MAB most commonly affects young CF patients[42, 43]. CF is a chronic disease that is caused by autosomal mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes for a widely distributed chloride channel in epithelial surfaces[44]. One of the main affected areas are the lungs, characterized by thick viscous mucus, impaired mucociliary clearance of inhaled bacteria and severe inflammation[45]. From an early age, CF patients have an increased susceptibility to airway bacterial infections, which can progress to chronic infections that can overtime lead to progressive deterioration of lung function. In fact, the most frequent cause of accelerated disease progression and mortality in patients with CF is due to chronic respiratory infections[45, 46].

In addition to defective mucociliary defense, hypersusceptibility to MAB infection may also be due to impaired innate immune response during the early stages of granuloma formation[47]. The CFTR appears to play an important role in neutrophil recruitment and granuloma formation and integrity, where loss of function was shown to promote rapid extracellular mycobacterial expansion.

3.3. Drug resistance

Infections by MAB are renowned for being difficult to treat, making it an “antibiotic nightmare” for clinicians. Low permeability of the cell wall and concentrated levels of efflux pumps poses as the primary obstacle to effective drug therapy[48]. In earlier studies, prior to differentiation of MAB from *M. chelonae*, the cell wall was shown to be 10-20 times less permeable to beta-lactams than MTB [49]. In mycobacteria, a family of proteins, called the MmpL and MmpS proteins, are present and typically act as transporters that regulate the permeability of the cell wall, where certain have been involved in drug effluxing. Compared to

MTB, MAB possesses twice as many annotated MmpL proteins than in MTB, 27 and 14 respectively[50]. Amongst the RGM, MAB was found to have the highest number of such proteins. Orthologs of MTB efflux pumps MmpS5/MmpL5 were found in MAB, sharing 56% and 65% sequence identify respectively[51]. In MAB, the drug export system increased the Minimal Inhibitory Concentration (MIC) to thiacetazone analogues, while in MTB it leads to clofazimine, bedaquiline and azole resistance.

Several other mechanisms of intrinsic resistance have been reported for MAB[48]. Notably upon exposure to macrolides, MAB can exhibit inducible resistance through expression of the erm41 gene, which encodes an erythromycin ribosome methyltransferase. The resulting enzyme methylates the 23s rRNA (encodes 50S ribosomal subunit) drug target of clarithromycin or azithromycin interfering with drug binding. Beta-lactams are inactivated in MAB through expression of the antibiotic-modifying beta-lactamases. Polymorphisms in the target gene EmbB causes MAB to be naturally resistant to ethambutol due the resulting disruption of drug-target binding[52]. Acquired mutations in the target genes of aminoglycosides and macrolides (particularly upon monotherapy of clarithromycin) have also been reported to confer high level resistance. Furthermore, WGS show evidence of lateral gene transfer between MAB and *Pseudomonas aeruginosa* and *Bulkholderia cepacia* in CF patients[53]. Genes characteristically found in *P. aeruginosa* and *B. cepacia* that are used for metabolism of aromatic compounds were discovered in MAB, while several MAB proteins related to intracellular survival in macrophages were found in both major CF pathogens.

4. Drug discovery for NTM

4.1. Approaches to drug discovery

The acquisition of antibiotic resistance in NTM by natural selection from residing in the environment has led to an urgent need for more effective drugs. The development of a successful antibiotic must meet several criteria of efficacy and safety. Additionally, it must have the ability to effectively reach the infected tissue and penetrate the host cell while maintaining potent inhibitory activity. The current drug pipeline for NTM is dry; there are currently no anti-NTM drugs in preclinical development[13].

In drug discovery, two general approaches can be distinguished. The first is termed *de novo* discovery where entirely new drug targets are identified in the desired pathogen. It requires extensive work using genetics, next generation sequencing, chemical biology and microbiology. Whole-cell screening or screening of a "random" compound library for the identification of novel targets are both time and money consuming, limited by the existing knowledge available of the microorganism of interest[13]. The second approach involves searching for new chemistry against a target that has already been validated. Analogues against the validated target are first screened against the pathogen, upon which x-ray crystallography can then be used for hit optimization. Using compounds with known anti-TB activity can accelerate NTM drug discovery and development. Previous studies have demonstrated that TB-active libraries generate significantly high hit rates for NTM[54]. In both approaches, compounds are tested for antimicrobial activity against the pathogen of interest and a reliable method is needed to measure bacterial growth and viability.

4.2. Challenges in NTM drug discovery

Drug discovery for NTM carries its own set of challenges. Historically, the disease-causing potential of NTM was neglected; research on drug resistance and mechanism of action largely focused on the professional pathogens MTB and *M. leprae*. Extensive knowledge of drug targets in NTM is currently lacking and represents a major barrier to *de novo* drug discovery[13]. As such, the approach of repurposing TB drugs and TB drug targets has been prioritized. A lack of an animal model to measure *in-vivo* outcomes further impedes on successful drug development.

Hit rates from primary screens for NTM are discouraging, where it can be lower than 0.1% for MAB[13]. The challenges are two-part: (1) difficulties in overcoming the innate features of NTM related to intrinsic drug resistance; (2) difficulties in measuring drug susceptibility in the laboratory[27]. Arguably, both essentially stem from a same single trait of NTM: their hydrophobic cell wall. Resistance mechanisms in NTM have been detailed above; the issue of drug susceptibility testing (DST) discrepancies will be further elaborated in upcoming sections. For now, it is worth mentioning that the waxy cell membrane is a prominent barrier to drug penetration and intracellular accumulation while also creating methodological challenges in the laboratory. The hydrophobicity promotes surface adherence and aggregation of cells making

reproducible DST difficult to achieve. Other laboratory barriers include inter-assay variability, where a lack of a standardized method to measure MIC *in vitro* can lead to discrepant results. In this project, we focused on exploring the latter and its consequences on NTM drug development.

5. Drug susceptibility testing for NTM

5.1. Methods for drug susceptibility testing

A crucial step in drug discovery requires testing the novel compounds for antimicrobial activity using a reliable measure of bacterial growth and viability. The degree of susceptibility or resistance of a microorganism to an antibiotic is reflected in the measurement of the MIC. In this study, we were interested in the MIC required to inhibit 90% or 99% growth, also known as MIC90 and MIC99 respectively. If inhibition of growth occurs at an antibiotic dose equal or lower than the MIC, the bacteria is susceptible to the drug. The measurement of MIC may be used for various purposes. Originally, DST served as diagnostic tool to differentiate NTM from MTB[55]. However, once the clinical relevance of NTM infections was established, DST was performed to guide antimicrobial therapy and allowed the monitoring of emerging drug resistance in cases of treatment failure. To date, DST is recommended for RGM but typically not for SGM. The Clinical and Laboratory Standards Institute (CLSI) guidelines recommend broth microdilution for most NTM species (broth macrodilution is recommended for MAC)[55, 56].

For the purposes of drug discovery, different assays are available to measure DST. The direct counting of colony-forming units (CFU) on solid media has been the longstanding gold standard in the field of bacteriology. Measuring optical density, or absorbance, according to the turbidity of a broth culture is also common, providing an indirect measure of growth. There are also commercially available microbial cell viability assays that measure the amount of a metabolite to indicate the degree of metabolic activity as a proxy of growth. Each assay inherently has pros and cons that will be further discussed in this paper.

5.2. Ongoing debate: *in vitro* phenotypic susceptibility versus *in vivo* treatment outcome

The role of DST in guiding NTM therapy remains unclear and controversial due to reported discrepancies between *in vitro* susceptibility testing and *in vivo* clinical outcomes. To date,

evidence supporting such a correlation only exist between MAC and macrolides and amikacin, and *M. kansasii* and rifampicin[33, 57]. It is recommended by the CLSI guidelines to perform DST on MAC isolates for clarithromycin and amikacin exclusively, particularly if the patient received macrolides in the past. Despite lack of empirical data, clinicians may still request susceptibility testing against other antibiotics. For RGM, correlations exist for extrapulmonary disease but remain elusive for pulmonary disease. Clinical guidelines recommend DST results to guide drug selection for MAB infection. In this case, established MIC thresholds are believed to be clinically useful for clarithromycin/azithromycin, amikacin and a few additional agents. Inducible macrolide resistance may arise upon sufficient drug exposure during treatment; however, this may not be initially captured when testing MIC *in vitro*[57]. As such, it is now standardized to test for clarithromycin susceptibility in the clinical microbiology lab for MAB over a prolonged period of two weeks.

Poor correlation between *in vitro* and *in vivo* observations may be due to differences in growth conditions of the bacteria. The environment within a host is significantly different from culturing the mycobacteria in a nutrient-rich broth[13]. The latter would provide optimal conditions for exponential growth such that drug action that is dependent on bacterial metabolism may have a higher potential to inhibit growth. Alternatively, infection in host lungs represent a stressful environment for mycobacteria. It is likely to undergo various physiological adaptations that may lead to reduced metabolic activity, which in turn reduces the bioavailability of the drug target leading to drug tolerance[13]. In pulmonary NTM, biofilm formation or the process of stationary growth phase or persistence may affect drug penetration and activity leading to phenotypic resistance[27]. To thrive under stressful conditions, the bacteria may also adopt strategies to either increase cell impermeability to foreign substances or exhibit higher effluxing of the antimicrobial compounds. Likewise, reduced antibiotic penetration into infection sites may lead to subtherapeutic concentrations to occur, rendering the treatment ineffective. Finally, hydrophobic NTM cells have a preference for adhering to the wells or tubes[27]. Cell suspension in broth media is not uniform and may hinder on the accuracy of turbidity measurement as a proxy of number of cells. The turbidity also increases proportionally to bacterial growth until during the early phases. Once the mid or late phase of growth is reached, turbidity disappears paired with the presence of further aggregation of cells. As such, we

explored in this project how MIC measurement based on turbidity compares with other cell viability assays that do not rely on cell mass.

6. Dihydrofolate reductase

Both prokaryotes and eukaryotes require reduced folates for the biosynthesis of DNA, RNA and proteins for cell growth and survival[58]. In humans, this is achieved by the salvage pathway where folate is acquired from diet. Contrary to humans, bacteria can produce reduced folate *de novo* through the essential enzyme dihydrofolate reductase (DHFR). The DHFR enzyme catalyzes the reduction of dihydrofolate to tetrahydrofolate in a NADPH-dependent manner. Tetrahydrofolate is a co-factor required for the synthesis of thymine, purine bases and amino acids (serine, glycine and methionine)[59].

Growth inhibition by targeting folic acid metabolism can be achieved through various mechanisms. The co-factor tetrahydrofolate is needed to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) by thymidylate synthase[60]. Inhibition of DHFR activity leads to an accumulation of dUMP, such that it will instead be converted into deoxyuridine triphosphate (dUTP) that will then incorporate into DNA and cause cell death[60, 61]. In mycobacteria, depletion of tetrahydrofolate was also found to disrupt the activated methyl cycle, reduce production levels of methionine and S-adenosylmethionine, and ultimately decrease production of mycolic acid precursors affecting cell wall integrity[59].

The emergence of drug-resistant MTB generated interest in targeting the folate pathway for drug development. Known inhibitors of the folate pathway in bacteria include trimetrexate, trimethoprim, sulfamethoxazole and the anti-MTB drug *para*-aminosalicylic acid (PAS)[59]. Trimetrexate is a competitive inhibitor of DHFR but has been found to be ineffective against live MTB due to poor cell permeability[62]. Trimethoprim is a broad-spectrum DHFR inhibitor that show low potency against MTB DHFR when administered as a single agent[63]. Its activity is potentiated through synergistic effects when paired with sulfamethoxazole in the drug co-trimoxazole (1:5 ratio)[64]. Upstream of DHFR, sulfamethoxazole targets dihydropteroate synthetase by acting as a structural analog of *para*-aminobenzoic acid[59]. Co-trimoxazole shows *in vitro* activity against MTB but further studies are needed to establish its efficacy for

MDR-TB treatment. The prodrug PAS inhibits both DHFR and flavin-dependent thymidylate synthase in mycobacteria[59]. According to the WHO 2019 guidelines, PAS can be used to complete a longer MDR-TB regimen in cases where other options such as bedaquiline, linezolid, clofazimine or delamanid are not possible[65]. In addition to bacteria, the folate pathway has been targeted against protozoa (e.g. malaria) as well as in chemotherapy[66]. Methotrexate acts as an anti-cancer agent by targeting human DHFR. Upon sequence alignment, human DHFR was found to share 26% sequence identity with MTB DHFR [67].

RATIONALE AND OBJECTIVE OF RESEARCH

Nontuberculous mycobacteria are emerging opportunistic pathogens that most frequently cause chronic, TB-like, pulmonary infections that are difficult to treat. One of the most clinically relevant, and arguably the most drug resistant NTM species is MAB. Patients undergo a prolonged multi-drug regimen with severe toxic effects and overall poor cure rates. Current drug development efforts are limited by the lack of extensive knowledge on NTM drug targets and challenges in NTM drug susceptibility measurement in the laboratory.

In this project, our objective is to discover new chemistry against MAB to improve upon currently available therapies. We hypothesize that TB drug targets offer the most direct path to new antimicrobial therapies for NTM pathogens, but that we need new chemical entities to act at these established targets. Through our research efforts, we also set out to examine inter-assay variability when measuring MIC in NTM to inform our approach to DST in guiding compound selection.

The aims of the project are as follows:

Aim 1: Evaluate the essentiality of the candidate drug target DHFR using CRISPR interference (CRISPRi) on isolates of MAB.
Aim 2 – Part 1: Screen novel TB-active DHFR inhibitors that are in pre-clinical development from the Structure-guided Drug Discovery Coalition (SDDC), to assess for antimicrobial activity in MAB.
Aim 2 – Part 2: Assess inter-assay variability in measuring MIC of amikacin, rifampicin and bedaquiline against MAB using agar dilution, OD600, BacTiter-Glo and AlamarBlue.

CHAPTER II

Validation of dihydrofolate reductase as a therapeutic target for the treatment of *M. abscessus* infection

This section is adapted from an article in preparation for publication.

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Manuscript in preparation.

1. Abstract

Nontuberculous mycobacteria (NTM) are emerging opportunistic pathogens that cause chronic, tuberculosis (TB)-like lung disease. One of the most clinically relevant species is *Mycobacterium abscessus* having the most detrimental impact on lung function and patient outcome. The success rate of treatment with current antibiotics is low (50% or less). This study aims to discover new drugs against this species, where we hypothesize that orthologues of TB drug targets would offer the most direct path to new antimicrobial therapies for NTM. We used a combination of CRISPRi and chemistry to test whether dihydrofolate reductase (DHFR) would be a valid target for the treatment of *M. abscessus*.

In preliminary work, the CRISPRi methodology has been first validated in the model organism *M. smegmatis* and subsequently in *M. abscessus*. With this technique, the DHFR gene, *dfrA*, was observed to be essential for *in vitro* growth of two subspecies of *M. abscessus*. A series of candidate DHFR inhibitors has been tested for *in vitro* growth inhibition using broth microdilution and conventional agar dilution method. A number of these compounds showed potent antimicrobial activity against *M. abscessus* (MIC90 at 10μM or lower). In this study, we also examined inter-assay variability when measuring MIC in NTM using the following assays: agar dilution, OD600, BacTiter-Glo and AlamarBlue. We found that highest reproducibility was achieved by agar dilution and OD600 while BacTiter-Glo acted as an outlier.

Together, these data suggest that DHFR can serve as a promising drug target for NTM infections, as shown via CRISPRi and chemical inhibition for *M. abscessus*. Our data also suggest that the OD600 assay is most suitable to guide compound selection for drug discovery against NTM.

2. Introduction

Nontuberculous mycobacteria are emerging opportunistic pathogens that can cause chronic, inflammatory, TB-like diseases. Approximately 65-90% of NTM disease cases are pulmonary, and acquisition of NTM is via inhalation and ingestion of organisms from environmental sources [2]. Importantly, NTM incidence in Canada as well as in the rest of the world has been increasing, where pulmonary NTM disease burden is now several-fold greater than that of TB [10]. Patients with pre-existing lung damage (e.g. the cystic fibrosis population), and immunosuppressed hosts (e.g. organ transplant recipients) have increased susceptibility to these infections. One of the most common and clinically relevant NTM species is MAB, having the most detrimental impact on lung function of patients [42]. Moreover, recent evidence of person-to-person transmission of MAB has emerged; suggesting that there is a risk of cross-infection that also needs to be controlled[4].

The success rate of treatment with antibiotics against MAB is low (30-50%) when compared to TB (over 95%). This lower treatment efficacy is largely due to the fact that these organisms are intrinsically resistant to many of the current anti-TB drugs and broad-spectrum antibiotics. To overcome this problem, treatment regimens are long (12-24 months), resulting in a greater risk of treatment-associated toxicity. Yet, even with these long, multidrug therapies, antibiotics often only suppress the infection, and relapse of infection commonly occurs after therapy is completed [68]. Despite the difficulties with treatment, coupled with the poor outcomes of therapy, there is limited NTM drug discovery work, and low therapeutic ratio of current drugs has caused reluctance among clinicians to offer therapy to all individuals with a positive culture [31]. New chemistry against MAB is urgently needed to improve patient outcomes and prevent further decline of lung function.

3. Methodology

Drugs used in this study

The DHFR inhibitors were donated by the SDDC, our collaborator on this project. In their previous work, the compounds were shown to exhibit potent activity against MTB in the sub-micromolar range. Amikacin (AMK), rifampicin (RIF), bedaquiline (BDQ) and isoniazid (INH) were purchased from Sigma-Aldrich.

Bacterial strains and culture conditions

The reference strains *M. abscessus* ATCC 19977, *M. avium* ATCC 35713, and *M. smegmatis* ATCC 700084 were grown in Middlebrook 7H9 broth (Difco) at 37°C. The media was supplemented with glycerol (0.2%), Tween80 (0.05%) and albumin dextrose catalase (ADC) (10%). Mycobacterial strains were grown on 7H10 agar plates supplemented with glycerol (0.5%) and oleic ADC (10%) and incubated at 37°C for up to three days. The media was supplemented with the appropriate antibiotics and doses as further described below.

Design and preparation of CRISPRi plasmid

The CRISPRi methodology was adapted from Rock et al. [69]. To achieve gene knockdown, the system utilizes a sgRNA designed against our gene of interest that guides a catalytically inactivated Cas9 nuclease into close proximity to the target being transcribed, thereby interfering with transcription. The CRISPRi construct is under the control of the TetR promoter such that targeted gene knockdown is inducible in the presence of anhydrotetracyline (ATC). The sgRNA is homologous to a 20-22 bp region of our target gene starting with a G or A and was designed to be 2 bp upstream of a protospacer adjacent motif (PAM) in the template strand of the gene (Table 1). The PAM sequence was identified with the highest predicted fold repression in the template strand (see Figure 1). The sgRNA consists of forward and reverse oligonucleotides each designed to have additional 4 nucleotides added to their 5' end that are complementary to the overhangs generated from BsmBI digestion (Table 2). The forward primer consisted of the 20-22 bp region (from the template strand) with a GGGA added upstream; the reverse primer consisted of the reverse complement of the 20-22 bp region to which AAAC was added upstream. Both oligonucleotides were then annealed (annealing buffer: 50 mM Tris pH 7.5, 50 mM NaCl and 1

mM EDTA) using the following program: 95°C for 2min, 0.1°C/sec to 25°C. The CRISPRi PLJR962 backbone was BsmBI-digested in NEBuffer 3.1 (New England Biolabs) overnight and gel purified using Gel Extraction Kit (Qiagen) by following the manufacturer's protocol. The annealed oligos was then ligated into the resulting CRISPRi backbone at room temperature (RT) overnight using T4 DNA ligase (Fermentas). The construct was transformed into DH5α competent *E. coli* cells through heat shock (30min/ice, 45 sec/42°C, 5min/ice, +SOC media, 1hr/37°C shaking). Plasmid-containing colonies were selected for on LB agar plates supplemented with 25 µg/ml kanamycin (kan). Plasmid was extracted from picked colonies (grown overnight in LB broth + 25 µg/ml kan) using Plasmid Miniprep Kit (Qiagen) and clones were sent for Sanger sequencing to confirm presence of CRISPRi target.

PAM Table

PAM	Fold repression	SD
5'-NNAGAAG-3'	216.7	10.0
5'-NNAGAAT-3'	216.2	10.4
5'-NNAGAAA-3'	158.1	22.8
5'-NNGGAAG-3'	145.2	5.3
5'-NNAGAAC-3'	120.5	7.9
5'-NNGGAAA-3'	110.5	26.4
5'-NNAGCAT-3'	84.6	5.2
5'-NNAGGAG-3'	82.2	9.2
5'-NNAGGAT-3'	64.7	8.7
5'-NNAGCAA-3'	53.4	9.9
5'-NNGGAAC-3'	51.5	6.2
5'-NNGGAAT-3'	47.3	3.3
5'-NNAGCAG-3'	42.2	7.0
5'-NNAGGAA-3'	38.5	5.2
5'-NNAGGAC-3'	25.5	0.8
5'-NNGGGAG-3'	24.7	1.9
5'-NNGGGAT-3'	24.2	3.4
5'-NNGGGAA-3'	12.3	0.8
5'-NNAGCAC-3'	11.9	1.2
5'-NNGGGAC-3'	7.9	1.0
5'-NNGGCAT-3'	6.7	0.9
5'-NNGGCAG-3'	4.0	0.3
5'-NNGGCAA-3'	3.3	0.3
5'-NNGGCAC-3'	2.7	0.3
ctrl sgRNA	1.3	0.1

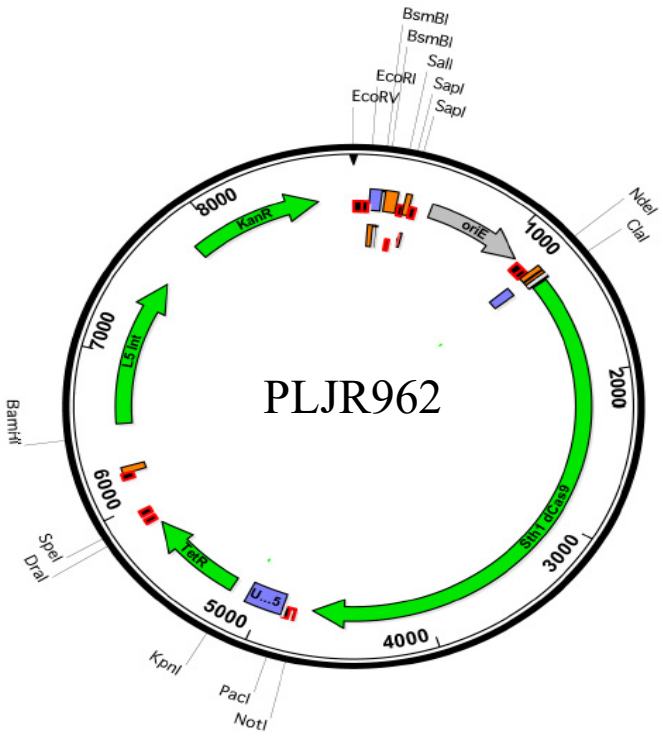

Figure 1. Table of PAM sequences with predicted fold repression and CRISPRi construct with PLJR962 backbone - Adapted from Rock et al. (2017).

Species	Gene name	Gene number	sgRNA targeting sequence (5' – 3')	PAM (5' – 3')
M. smegmatis	*lppz*	MSMEG_2369	TCGGCACGGGGTGCGCCCGGT	NNAGCAT
	rpoB	MSMEG_1367	CCGTCGAGGACATGCCGTTC	NNAGGAT
	folA	MSMEG_2671	GGCGTCTGCCCGAGGACCTC	NNGGAAT
	DHFR gene	MSMEG_0308	GGCGACGTCGCCGAACTGCACC	NNAGAAC
M. abscessus	*mmpL4b*	MAB_4115c	CTGAGTCTGCAAAGCGCTGGAC	NNAGAAG
	rpoB	MAB_3869c	GGCTCGATGTCGCTGTCGTT	NNAGAAG
	dfrA	MAB_3090c	CGTTGGCACAACGCTGCGAAGT	NNAGAAA

Table 1. sgRNA targeting sequences and PAM sequences used for CRISPRi plasmid design.

Target	Primer sequence	Use
MSMEG_2369	GGGAACCGGGCGCACCCCGTGCCGA	CRISPRi (Forward primer)
	AAACTCGGCACGGGGTGCGCCCGGT	CRISPRi (Reverse primer)
	TGACGGGTGCGATCAAAGAG	qRT-PCR (Forward primer)
	CAACCGGTCCTGGATGTACG	qRT-PCR (Reverse primer)
MSMEG_1367	GGGAGAACGGCATGTCCTCGACGG	CRISPRi (Forward primer)
	AAACCCGTCGAGGACATGCCGTTC	CRISPRi (Reverse primer)
	AGTGGTGAACCGTTCCCGTA	qRT-PCR (Forward primer)
	ACATTCCATCTCGCCGAAGC	qRT-PCR (Reverse primer)
MSMEG_2671	GGGAGAGGTCCTCGGGCAGACGCC	CRISPRi (Forward primer)
	AAACGGCGTCTGCCCGAGGACCTC	CRISPRi (Reverse primer)
	ATCTATGCGATGGCACTGGC	qRT-PCR (Forward primer)
	GTACTGGTCTGCCACTCACC	qRT-PCR (Reverse primer)
MSMEG_0308	GGGAGGTGCAGTTCGGCGACGTCGCC	CRISPRi (Forward primer)
	AAACGGCGACGTCGCCGAACTGCACC	CRISPRi (Reverse primer)
	GGTAAGGATGTGTGGGTGGT	qRT-PCR (Forward primer)
	ACCCACTCCGAACGCAT	qRT-PCR (Reverse primer)
MSMEG_2758 (*sigA*)	AAAACCATCTGCTGGAGGCG	qRT-PCR (Forward primer)
	TGGCGTAGGTCGAGAACTTG	qRT-PCR (Reverse primer)
MAB_4115c	GGGAGTCCAGCGCTTTGCAGACTCAG	CRISPRi (Forward primer)
	AAACCTGAGTCTGCAAAGCGCTGGAC	CRISPRi (Reverse primer)
	AGCATCGCACTCTTTGACGA	qRT-PCR (Forward primer)
	GCCATCCAACGCATCGAAAA	qRT-PCR (Reverse primer)
MAB_3869c	GGGAAACGACAGCGACATCGAGCC	CRISPRi (Forward primer)
	AAACGGCTCGATGTCGCTGTCGTT	CRISPRi (Reverse primer)
	GAGGCGTTGCTGGACATCTA	qRT-PCR (Forward primer)
	GTTCACCTTGTACCGACCCA	qRT-PCR (Reverse primer)
MAB_3090c	GGGAACTTCGCAGCGTTGTGCCAACG	CRISPRi (Forward primer)
	AAACCGTTGGCACAACGCTGCGAAGT	CRISPRi (Reverse primer)
	CGGAGAGATCTATCGGCTGT	qRT-PCR (Forward primer)
	AATTGATAGCGCAAGCCGGA	qRT-PCR (Reverse primer)
MAB_3009 (*sigA*)	CTCAACGCCGAAGAGGAAGT	qRT-PCR (Forward primer)
	TTGGCTTCCAGCAGATGGTT	qRT-PCR (Reverse primer)
cas9	CTACGCCCAGATCGTGAAGG	qRT-PCR (Forward primer)
	TCGGGAACACGTTGATCAGG	qRT-PCR (Reverse primer)
sgRNAID	TTCCTGTGAAGAGCCATTGATAATG	Sequencing clones (plasmid PLJR692)
pPLJR962	CTCGCGATAATGTCGGGCAA	PCR (Forward primer) (check for presence of plasmid)
	CTCACCGAGGCAGTTCCATA	PCR (Reverse primer)

Table 2. Primers used for CRISPRi, qRT-PCR and PCR to investigate essentiality of the DHFR gene in NTM.

Electroporation conditions

The CRISPRi plasmid was electroporated into mycobacteria as follows. The bacterial culture was first grown in 60 ml of 7H9 to OD_{600} of 0.5-0.8 after which it was split into two 50 ml conical tubes and centrifuged at 4,000 RPM for 15 min at 10C. The supernatant was discarded, and the pellet was resuspended in 35ml of cold 10% glycerol (stored at 4°C). Centrifugation was repeated, supernatant discarded, and pellet was washed with 15 ml of 10% glycerol. The glycerol wash was repeated for a third time with 7.5ml of 10% glycerol and the pellet was resuspended into a final volume of 800 µl of 10% glycerol. A 200 µl volume of culture was added directly unto 500 ng of plasmid DNA in a 2-mm electroporation cuvette (Thermo Scientific). The cuvette was flicked gently twice on each side to ensure proper mixing and was rested on ice for 10 min. Electroporation was performed using GenePulser Xcell (Bio-rad): voltage 2500V, resistance: 1000 Ω, capacitance: 25 µF, and time constant 25 ms. Immediately after electroporation, 800 µl of pre-warmed 7H9 was gently added to the cuvette, mixed by inversion and incubated at 37°C overnight for recovery. Strains were plated on 7H10 + kan (25 µg/ml for *M. smegmatis* and 50 µg/ml for MAB) with and without ATC (0.1 µg/ml for *M. smegmatis* and 10 µg/ml for MAB) and bacterial growth was monitored as a function of interference of the gene targets. A total of 10 colonies were picked from the 7H10+kan (without ATC) plate per CRISPRi target and grown in 3 ml of 7H9+kan (at same doses as 7H10). DNA was extracted by preparing boiled lysate where bacterial pellets were resuspended in 100 µl dH_2O and incubated at 95°C for 20 min. PCR and gel electrophoresis on a 1% agarose gel containing ethidium bromide was performed to confirm presence of plasmid. The level of gene knockdown achieved was quantified by qRT-PCR.

RNA isolation and qRT-PCR

Strains containing the CRISPRi plasmid were grown in 10 ml of 7H9+kan to log phase (OD_{600} 0.5-0.8) and diluted into 60 ml of 7H9 to OD_{600} 0.05. This was split into two 30 ml cultures, one of which did not receive ATC while the other did (doses indicated above). The cultures were incubated at 37°C for 3.5 replication times until early log phase (OD_{600} 0.2-0.3) was reached. Cells were harvested by centrifugation and pellets were resuspended into 1 ml of TRIzol Reagent (Ambion). Cells were lysed by bead-beating using acid-washed glass beads (Sigma-Aldrich) three times at maximum speed for 30 seconds using Fastprep-24 (MP Biomedicals) and cooled on ice

for 3 min in between runs. Samples were incubated at RT/5min and centrifuged 12,000 x g/1min. The TRIzol layer was transferred into 200 µl chloroform:isoamyl 24:1 (Sigma-Aldrich), shaken vigorously, incubated at RT/3min and centrifuged 12,000 x g/15min. The aqueous phase was precipitated with 500 µl of isopropanol/1hr, centrifuged 12,000 x g/30min, washed twice with 1ml 70% EtOH (Sigma-Aldrich), air-dried and re-dissolved in 90µl RNAse-free water. A volume of 2.5 µl RiboLock (Fermentas) was added and RNA was dissolved for 30min at 4°C. Samples underwent Turbo DNAse treatment and RNA was purified using the Qiagen RNease Kit. This was followed by a second Turbo DNAase treatment and DNAse Inactivation Reagent (Thermo Fisher Scientific) was added, upon which RNA was isolated after centrifugation at 12,000 x g/1:30min. RevertAid M-MuLV Reverse Transcriptase (Thermo Fisher Scientific) was used to synthesize first-strand cDNA from 300 ng of RNA following the manufacturer's protocol. Relative gene expression was analyzed in triplicates by qRT-PCR using Maxima SYBR/Fluorescein qPCR Master Mix (Fermentas) in a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems), run on a 7300 Real-Time PCR System (Applied Biosystems). The sigA gene was used as a housekeeping gene to normalize the data.

Determination of MICs

The MICs were determined using agar dilution and three broth microdilution methods (OD_{600}, AlamarBlue and BacTiter-Glo). Cultures were grown in 7H9 to log phase OD_{600} 0.5-0.8 (approximately 2 X 10^7 CFU/ml)[70]. For agar dilution, log-phase culture (assessed by OD_{600}) was 10-fold serially diluted, 10^{-5} dilution was plated onto 7H10 agar, and 10^{-3} dilution was plated onto antibiotic-containing 7H10 plates at various doses. Plates were incubated for up to 3 days at 37°C. MIC99 by agar dilution was defined as a 2 $\log_{10}$ reduction in the number of CFU relative to the 10^{-5} plate. For the broth assays, log-phase culture was diluted to OD_{600} 0.005 (approximately 1.57 X 10^5 CFU/ml) with 7H9, in agreement with the literature [70-74]. Two-fold serial dilutions of antibiotics were prepared separately and 20 µl of each dose was added to 180 µl of diluted bacteria in a 96-well plate. Serial dilutions of the antibiotics were as followed: SDDC compounds (0.15 to 20 µM) using 7H9/10%DMSO (Sigma-Aldrich), AMK (0.053 to 110 µM) with 7H9, RIF (0.0093 to 20 µM) with 7H9 and BDQ (0.0017 to 3.6 µM) with 7H9 broth. Positive controls were AMK (55 µM), RIF (10 µM) and BDQ (10 µM), and negative control was INH (200 µg/ml) alongside a drug-free control. Plates were incubated at 37°C for 2 or 3 days for

MAB (depending on experimental condition as will be later described) and 4 days for *M. avium*, as previously reported in the literature[54, 71]. Either 20 µl of AlamarBlue (Bio-rad) and 12.5% Tween80 (Sigma-Aldrich) was added and incubated for 4 hrs/37°C or 20 µl of BacTiter-Glo (Promega) was added and incubated for 5-30 min/RT (depending on experimental condition). OD_{600}, fluorescence (excitation/emission of 530/590 nm) and luminescence were read on an Infinite M200 Pro Tecan plate reader. For the screening of SDDC compounds, a MIC90 was defined as the lowest concertation that reduced bacterial viability to 10%. For AMK, RIF and BDQ, a theoretical MIC99 was calculated by nonlinear fitting bacterial growth data to the Gompertz equation using GraphPad Prism 7.

4. Results

4.1. CRISPRi gene-knockdown reveals DHFR gene essentiality in NTM

To investigate the essentiality of the DHFR gene in NTM, the CRISPRi methodology was first validated in the model organism *M. smegmatis*. Two copies of DHFR, *folA* and MSMEG_0308, were identified from the online KEGG database. The CRISPRi construct was induced with 0.1 µg/ml ATC. Relative quantification of gene expression by qRT-PCR revealed that knockdown was achieved for both copies of DHFR (significantly higher for *folA*) upon induction with ATC compared to "No ATC" control (Figure 2). Effect on growth was verified upon knocking down nonessential gene control *lppz*, essential gene control *rpoB* (>99% growth suppression), *folA* (>99% growth suppression) and MSMEG_0308 (Figure 3). We inferred that f*olA* is an essential gene in *M. smegmatis*.

We then pursued CRISPRi-mediated gene knockdown in MAB however our initial attempts at inducing the system with 0.1 µg/ml ATC failed. Given that MAB has a tetracycline-modifying monooxygenase, we hypothesized that a higher dose of ATC (a derivative of tetracycline) would be needed to sufficiently induce the CRISPRi system. Prior to repeating CRISPRi with a higher dose of ATC, we investigated its effect on MAB growth. At the highest dose used, we did not see direct inhibition of growth. We therefore performed CRISPRi in MAB with 10 µl/ml ATC for the remainder of the study (Figure 4). We knocked-down the nonessential gene control *mmpL4b*, essential gene control *rpoB* and *dfrA* (MAB reportedly has a single copy of the DHFR gene). CRISPRi-mediated gene knockdown of *mmpL4b*, *rpoB* and *dfrA* in *M. abscessus* occurred in a target-specific manner with increasing expression of *Cas9* when dose of ATC is increased (Figure 5). Upon induction of *Cas9*, we observed suppression of transcripts of our target mRNA species: *rpoB* (90%), *mmpL4b* (95%) and *dfrA* (>99%). Phenotypic growth suppression on 7H10 plates were estimated as the following: *rpoB* (99%), *mmpL4b* (50%) and *dfrA* (>99%) (Figure 6). CRISPRi was repeated in *M. abscessus* subsp. *massiliense* where approximately >99% growth suppression occurred upon knocking down *dfrA*. We inferred that *dfrA* is essential for *in vitro* growth of MAB.

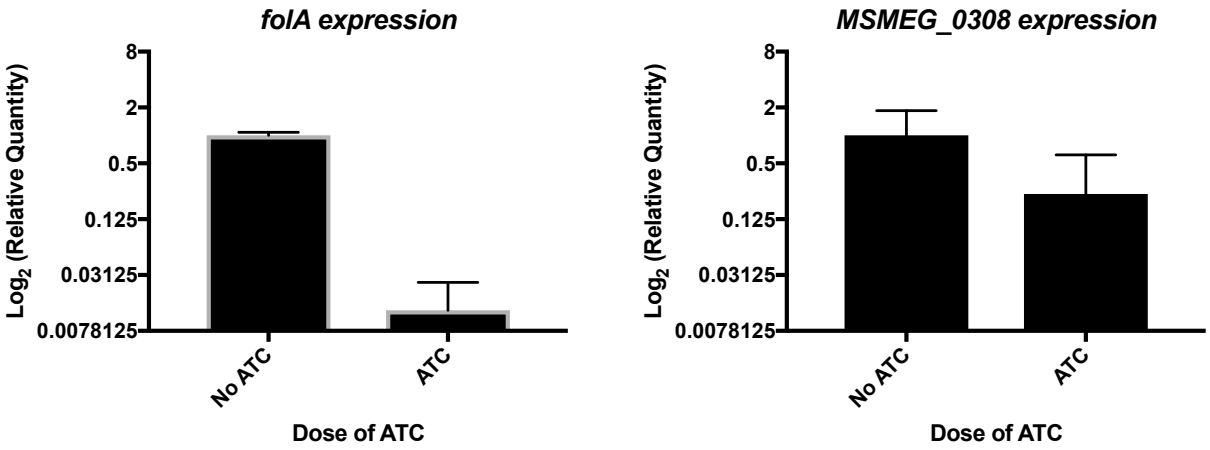

Figure 2. Gene knockdown by CRISPRi of *folA* and MSMEG_0308 in *M. smegmatis* was achieved. CRISPRi-mediated gene knockdown of *folA* (DHFR gene) and MSMEG_0308 (duplicate copy of DHFR gene) in *M. smegmatis* ATCC 700084 strain was quantified by qRT-PCR. Fold change in gene expression when inducing CRISPRi knockdown system with 0.1 µg/ml of ATC was assessed relative to the "no ATC control". Error bars are 95% confidence intervals of three technical replicates.

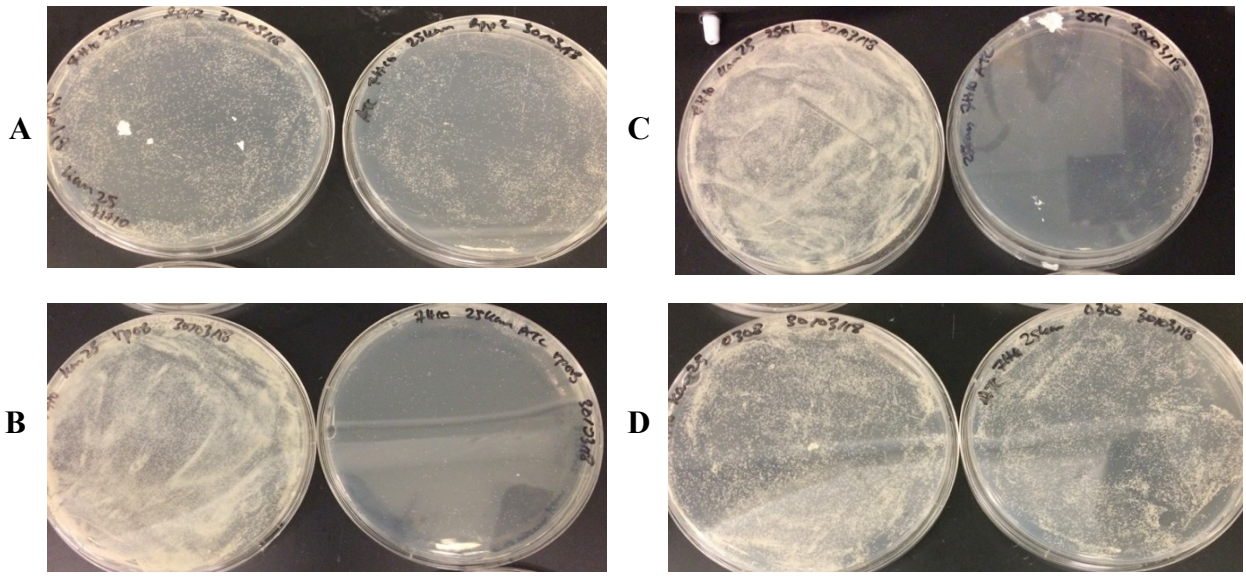

Figure 3. The DHFR gene *folA* is essential for *in vitro* growth of *M. smegmatis*.
CRISPRi-mediated gene knockdown was performed on *M. smegmatis* ATCC 700084.
A) Non-essential gene control (*lppz*) as a positive growth control, **B)** essential gene control (*rpoB*), **C)** DHFR gene (*folA*) and **D)** duplicate copy of DHFR gene (MSMEG_0308).
Transformants were plated on 7H10 agar plates + kan (25 µg/ml).
Left: no ATC; Right: 0.1 µg/ml ATC present. Pictures were taken three days after plating.

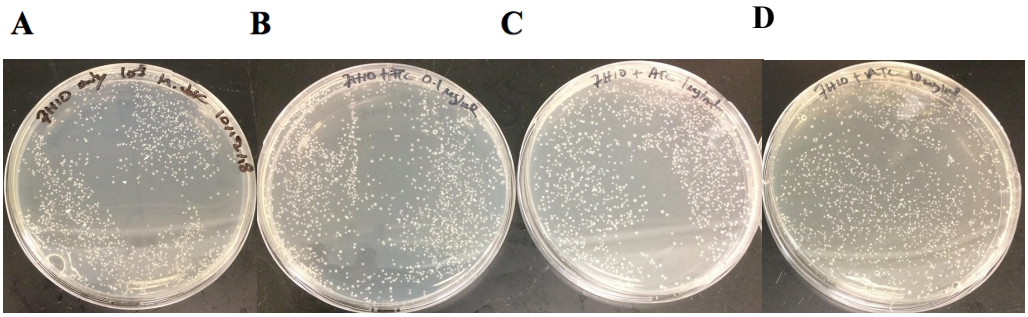

Figure 4. Higher dose of ATC does not inhibit growth of *M. abscessus*. A culture of *M. abscessus* ATCC 19977 was grown to mid-log phase and plated on ATC-containing 7H10 agar plates. **A)** 7H10 media only, **B)** 7H10 + 0.1 µg/ml ATC, **C)** 7H10 + 1 µg/ml and **D)** 7H10 + 10 µg/ml ATC. Pictures were taken three days after plating.

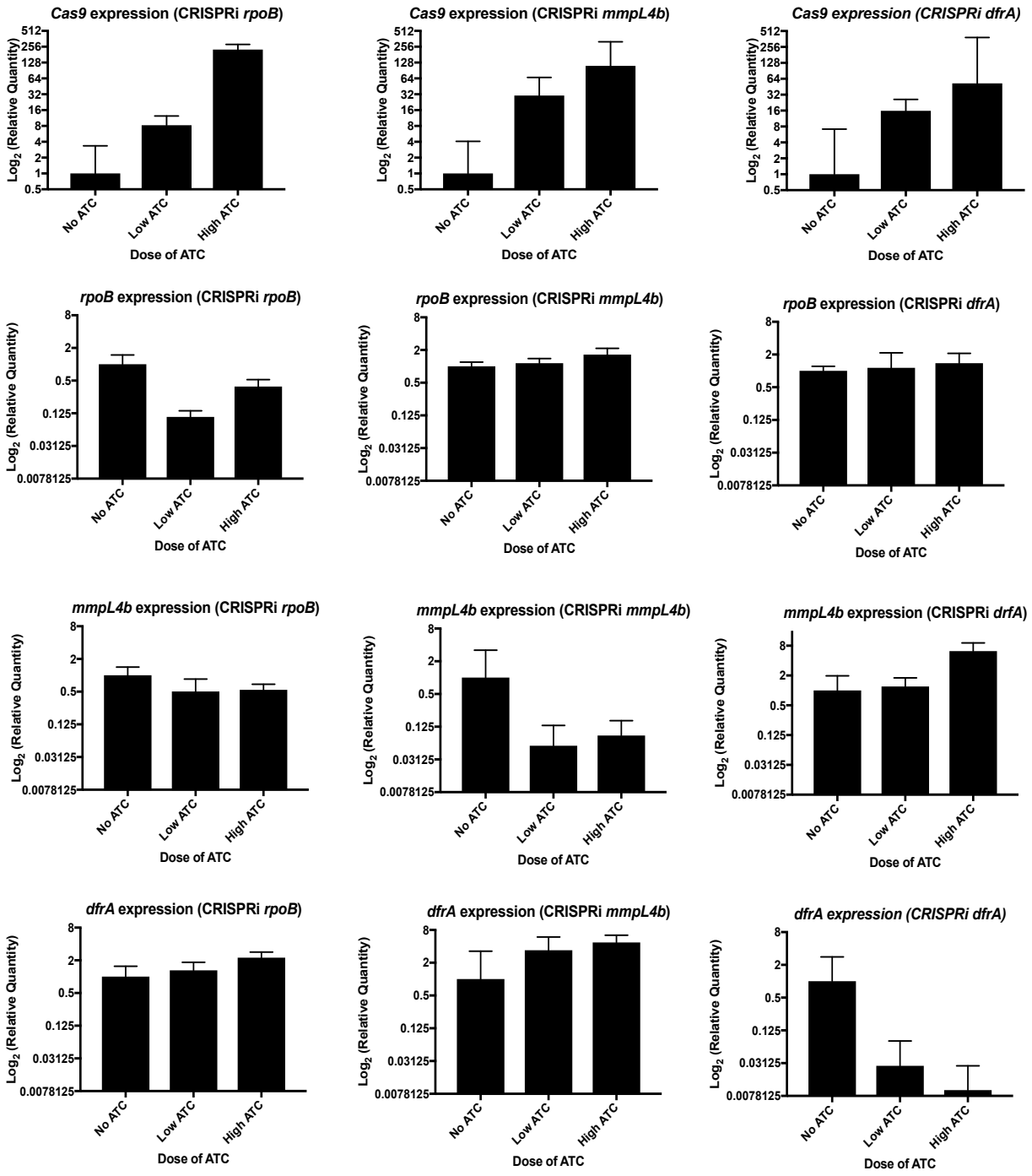

Figure 5. Gene knockdown by CRISPRi of *rpoB*, *mmpL4b* and *drfA in M. abscessus* occurred in a target-specific manner with increasing expression of *Cas9* when dose of ATC is increased. CRISPRi-mediated gene knockdown of *rpoB* (essential gene control), *mmpL4b* (non-essential gene control) and *dfrA* (target gene of interest) in *M. abscessus* ATCC 19977 strain was quantified by qRT-PCR. Fold change in gene expression when inducing CRISPRi knockdown system with a "low dose" (0.1 μg/ml) and "high dose" (10 μg/ml) was assessed relative to the "no ATC control". *Cas9* expression increased in an ATC dose-dependent manner. Gene knockdown of CRISPRi targets was best achieved for *dfrA*, but also achieved with *rpoB* and *mmpL4b*. Target-specificity was generally observed. Error bars are 95% confidence intervals of three technical replicates.

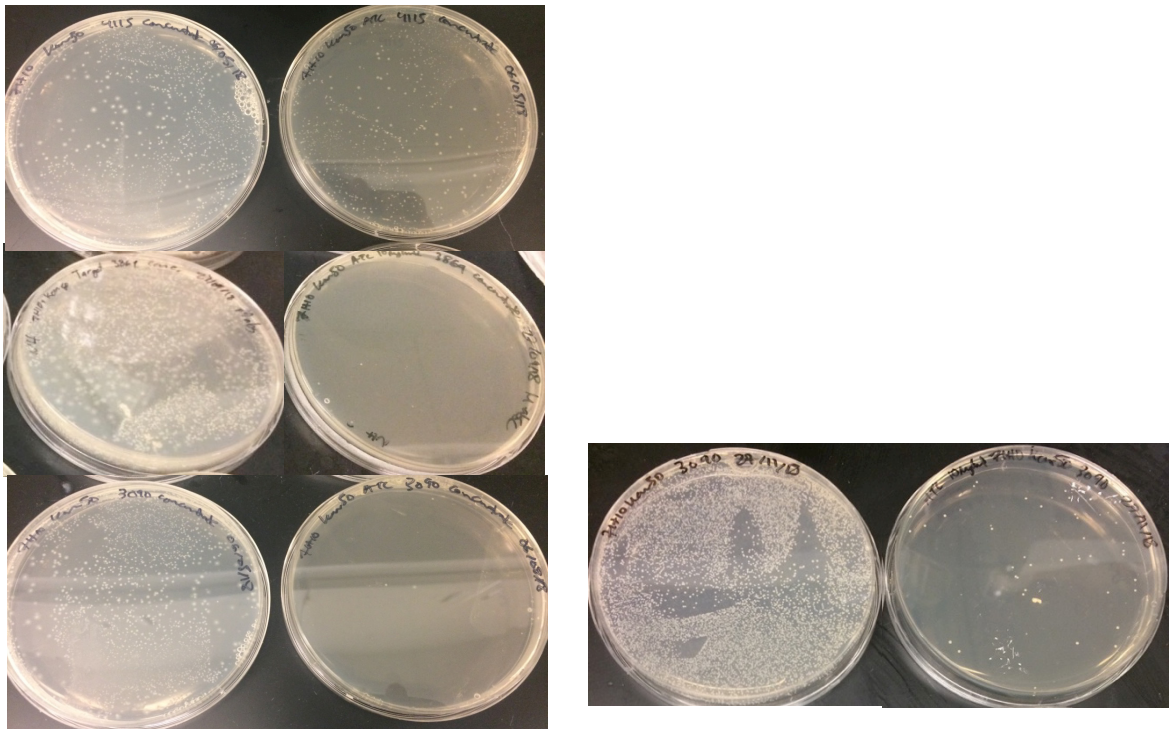

Figure 6. The DHFR gene *dfrA* is essential for *in vitro* growth of *M. abscessus*. CRISPRi-mediated gene knockdown was performed on *M. abscessus* subsp. *abscessus* (ATCC 19977) and *M. abscessus* subsp. *massiliense*. **A)** Non-essential gene control (*mmpL4b*) as a positive growth control, **B)** essential gene control (*rpoB*) and **C)** DHFR gene (*dfrA*). Transformants were plated on 7H10 agar plates + kan (50 µg/ml). Left: no ATC; Right: 10 µg/ml ATC present. Pictures were taken three days after plating.

4.2. Preliminary screening of DHFR inhibitors show antimicrobial activity against NTM

At the beginning of this project we received a first set of DHFR inhibitors from the SDDC and screened them against ATCC 19977 of MAB using the BacTiter-Glo assay. We found that only SDDC 610 and SDDC 1236 showed some level of inhibitory effect (less than 50%) (Figure 7). Prior to performing further drug screening with the laboratory strain of MAB, we were interested in comparing its drug susceptibility patterns to clinical isolates. As a proof-of-concept, we tested all eight SDDC compounds at 10 and 5 µM against two clinical isolates of the subspecies *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*. We found that the clinical isolates were susceptible to additional compounds compared to ATCC 19977 notably SDDC 781 and 1198 (Figure 7). Compound SDDC 610 was overall slightly more active against the clinical strains while SDDC 1236 was active against MT15-4079 but not for MT14-2546. Given the results, ATCC 19977 may arguably be considered representative of highly resistant MAB

clinical strains. For practicality purposes, the laboratory strain was deemed acceptable for preliminary drug screening and was used for the remainder of the study. As a further attempt to investigate the potential of DHFR as a novel anti-NTM target, we compared the antimicrobial activity of our eight compounds of MAB to that of *M. avium* ATCC 35713, a second clinically relevant NTM species. We found that compounds showing poor activity against MAB were significantly more effective at inhibiting growth of M. avium where >90% inhibition occurred for seven out of the eight compounds at 0.63-10 µM (Figure 8).

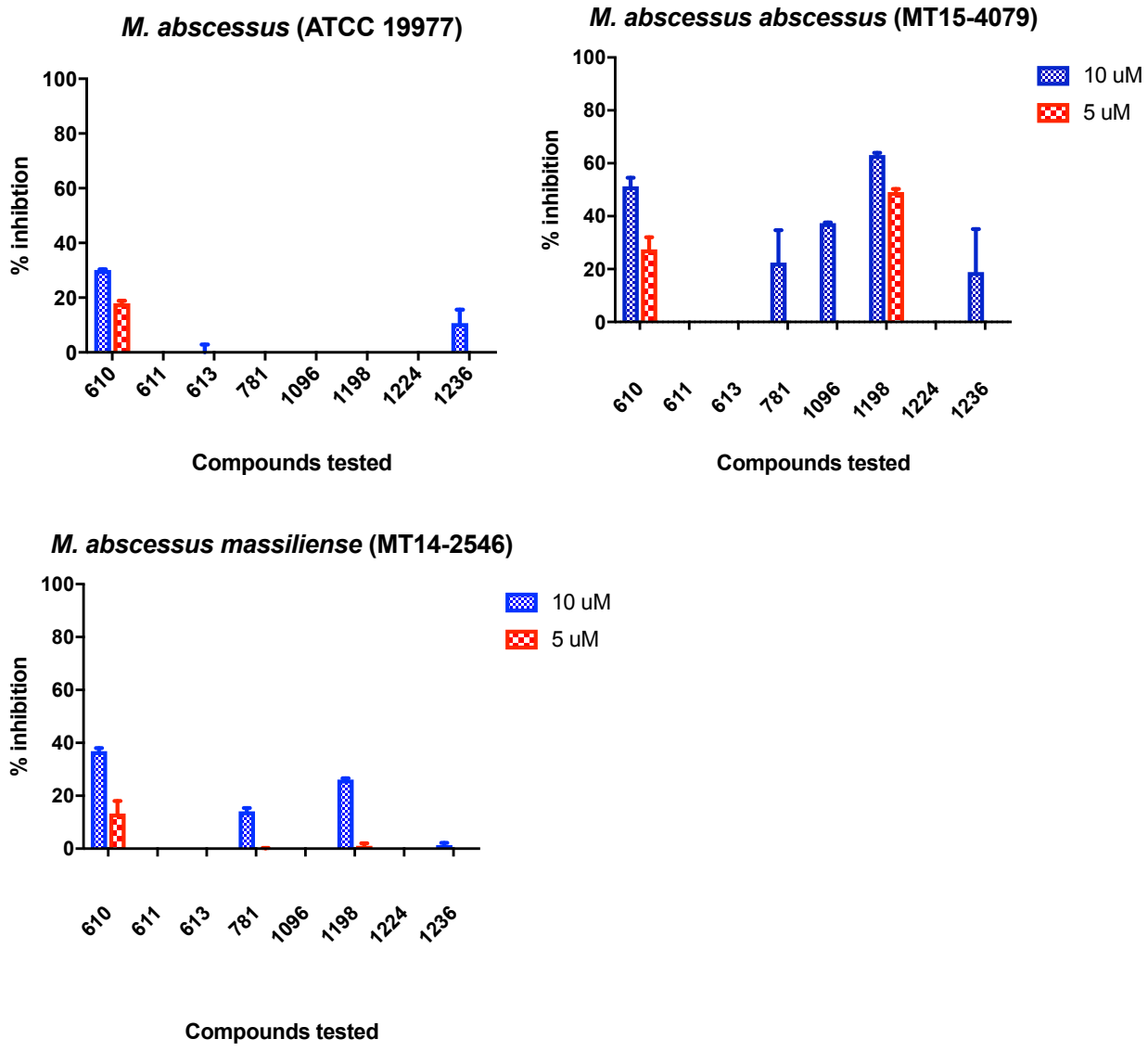

Figure 7. Inhibitory activity was achieved by DHFR inhibitors against laboratory and clinical strains of *M. abscessus.* Eight novel DHFR inhibitors in pre-clinical development were tested against the laboratory strain ATCC 19977 and two clinical isolates of *M. abscessus* at 10 and 5 µM. Inhibition of growth was assessed using the microbial cell viability assay BacTiter-Glo. Results are shown as the mean and standard deviation of technical duplicates.

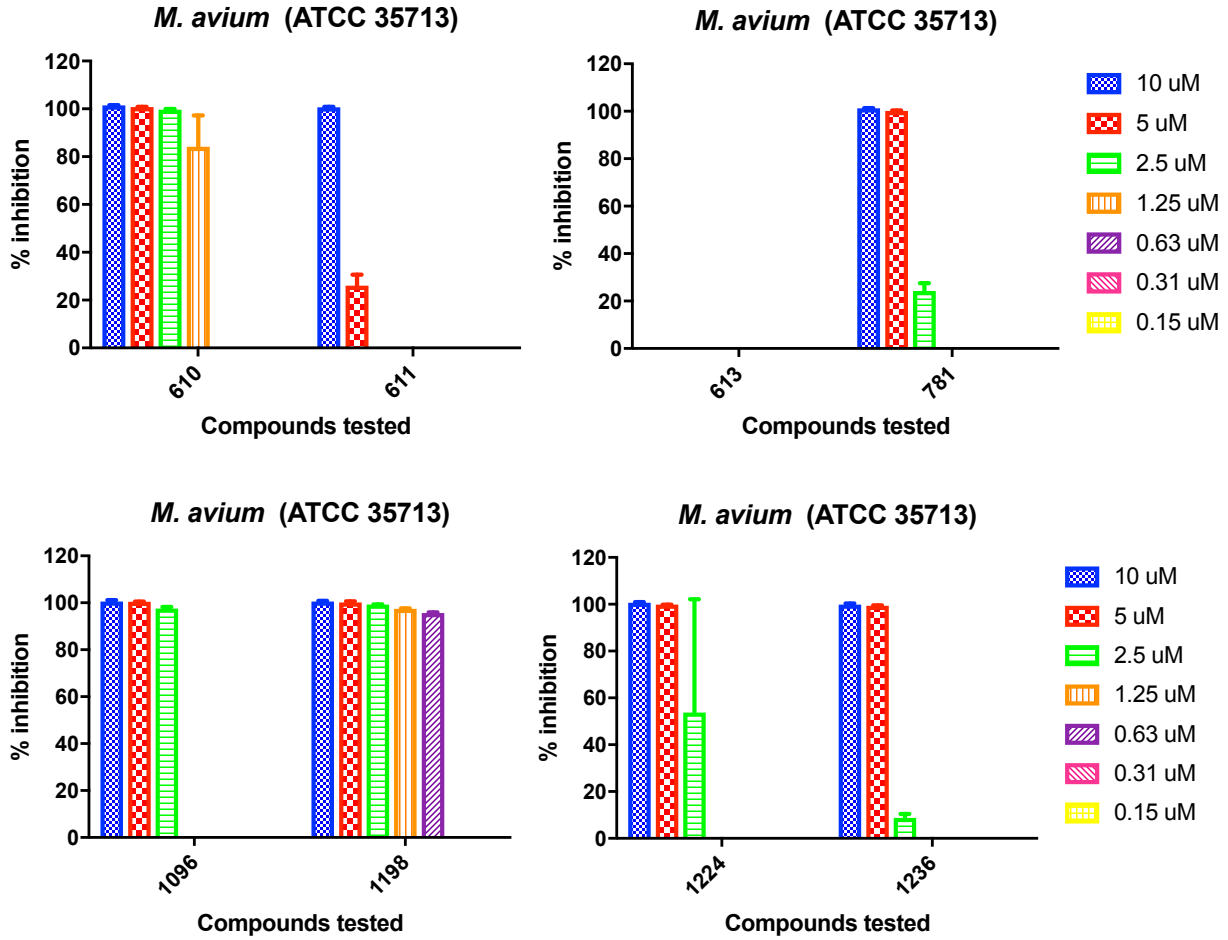

Figure 8. Inhibitory activity was achieved by DHFR inhibitors against laboratory strain of *M. avium*. Eight novel DHFR inhibitors in pre-clinical development were tested against the laboratory strain ATCC 35713 of *M. avium* at 0.15 to 10 µM. Inhibition of growth was assessed using the microbial cell viability assay BacTiter-Glo. Results are shown as the mean and standard deviation of technical duplicates.

4.3. Further screening of DHFR inhibitors using different assays yield discordant results

Our collaborator provided us with additional DHFR inhibitors (SDDC 1222, 1245 and 1250) that we screened alongside SDDC 610, 613 and 1236 against ATCC 19977 MAB using BacTiter-Glo (from here on, we report our findings in % viability). MIC90 was achieved with SDDC 1222 and 1245 at 5 and 10 µM respectively, while some level of inhibition occurred for compounds 610 and 1236 (Figure 9). From this, we were interested in cross-validating our results using

AlamarBlue and the conventional agar dilution method. Approximately 100% viability was observed at the highest doses of all compounds tested with AlamarBlue compared to 10% viability achieved by the positive control AMK (Figure 10). The agar dilution method was performed for our two most promising hits (SDDC 1222 and 1245) up to 20uM. While a reduction in growth was apparent at 10 and 20 µM, MIC90 was not achieved (Figure 11). Given these discrepant results, we decided to repeat the preliminary screening of our full set of DHFR compounds at 10 µM using the OD_{600} assay as growth can be monitored by the naked eye. MIC90 was achieved by SDDC 1096, 1198, 1222 and 1230 (Figure 12). For compounds 610, 781, 1236, 1245 and 1250, % viability was approximately between 11-20%. Compound 613 consistently showed an absence of inhibitory effect across all three broth assays tested against MAB (Figure 9, 10 and 12).

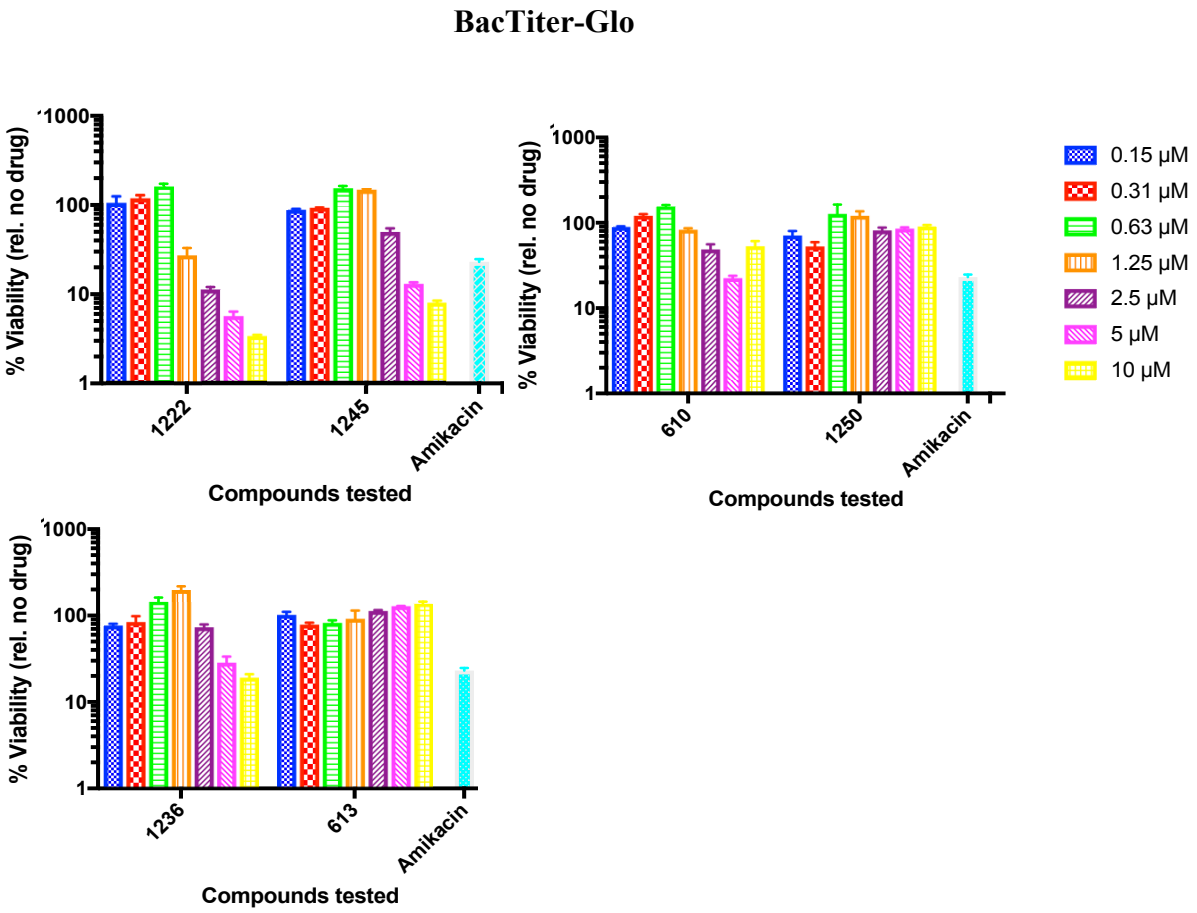

Figure 9. Inhibitory activity was achieved by DHFR inhibitors against *M. abscessus* ATCC 19977 by BacTiter-Glo. Additional DHFR inhibitors were tested against *M. abscessus* ATCC 19977 at 0.15 to 10 µM. AMK was included as a positive control at 55 µM. Percent cell viability was assessed using the microbial cell viability assay BacTiter-Glo. Results are shown as the mean and standard deviation of technical triplicates.

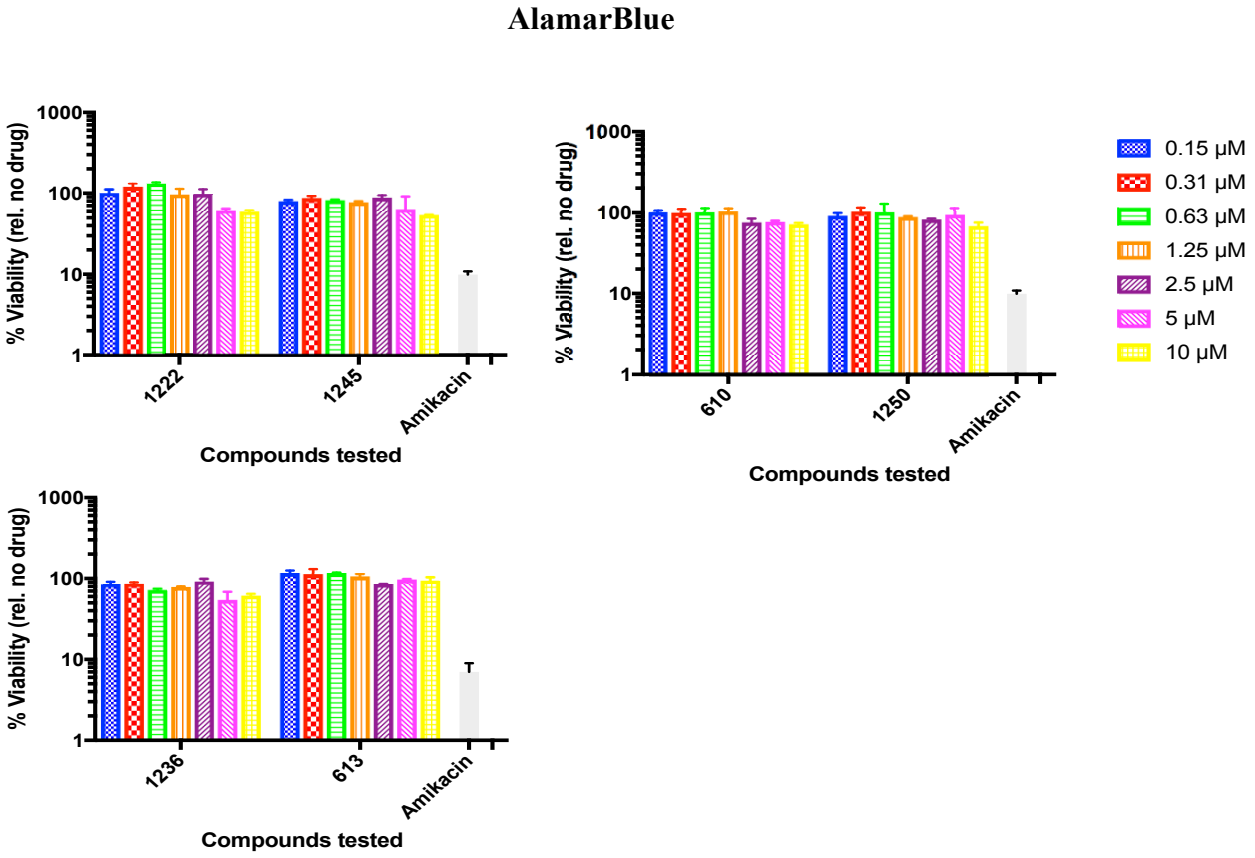

Figure 10. Inhibitory activity was achieved by DHFR inhibitors against *M. abscessus* ATCC 19977 by AlamarBlue. DHFR inhibitors were tested against *M. abscessus* ATCC 19977 at 0.15 to 10 µM. AMK was included as a positive control at 55 µM. Percent cell viability was assessed using the AlamarBlue assay. Results are shown as the mean and standard deviation of technical triplicates.

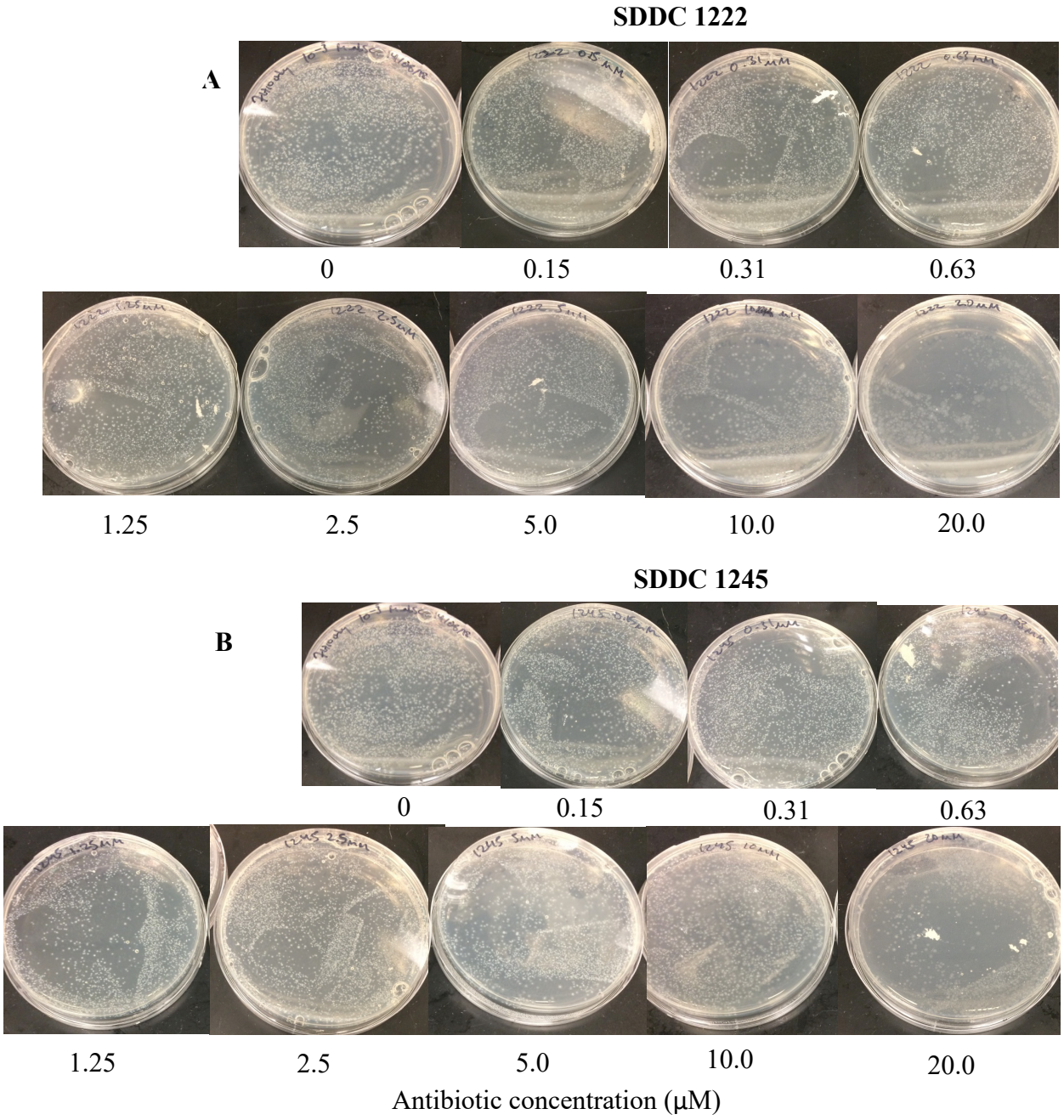

Figure 11. MIC99 was not achieved for SDDC 1222 and 1245 by agar dilution. DHFR inhibitors were tested against *M. abscessus* ATCC 19977 at various doses (0 to 20 μM) in 7H10 agar. Pictures were taken three days after plating.

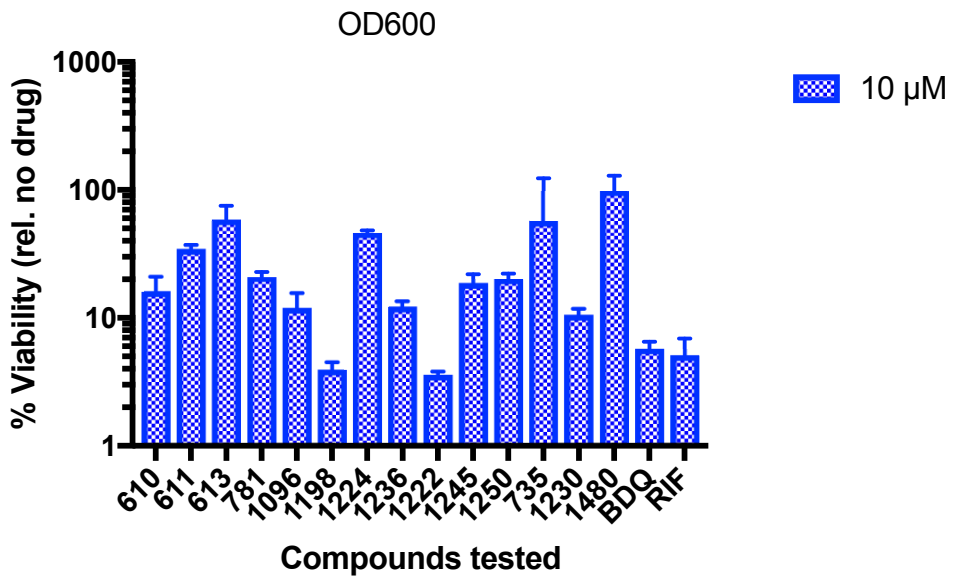

Figure 12. Inhibitory activity was achieved by DHFR inhibitors against *M. abscessus* ATCC 19977 by OD600. A series of DHFR inhibitors were tested against *M. abscessus* ATCC 19977 at 10 µM. BDQ and RIF were included as a positive control at 10 µM. Percent cell viability was assessed using OD600 readings. Results are shown as the mean and standard deviation of technical triplicates.

4.4. DST of AMK and RIF show reproducible inter-assay variability trends

Given the inter-assay variability in MIC90 values observed from our screening of DHFR inhibitors, we were interested in comparing the assays using two known anti-TB drugs AMK and RIF. We examined the reproducibility of each assay by repeating DST thrice for each broth assay and twice using agar dilution. OD_{600} and agar dilution showed the highest reproducibility, followed by AlamarBlue and BacTiter-Glo (Figure 13-16; Table 3). Higher doses of AMK and RIF consistently did not yield MIC90 when using the BacTiter-Glo assay.

4.5. BacTiter-Glo acts as an outlier regardless of short or long reagent incubation time

We investigated whether varying the incubation time of the culture with the BacTiter-Glo reagent would affect the DST results. On the same day the experiment was setup, an additional plate for OD_{600} readings was prepared acting as a reference. Readings were carried after 5 min and close to 30 min incubation with the reagent (we did not go over 30 min as recommended by

the manufacturer). BacTiter-Glo yielded higher % viability values overall compared to OD_{600} irrespective of the incubation time (Figure 17).

4.6. Inter-assay variability trends persist between 2- and 3-days treatment time

We examined whether DST results showed less variability during early phase of growth after two days incubation versus a separate MIC plate that incubated for three days. We assessed inter-assay variability for both 96-well plates for AMK, RIF and BDQ based on the MIC99 generated from fitting growth data to the Gompertz equation. We found that for all three drugs the inter-assay variability trends persisted between both conditions; BacTiter-Glo continued to act as an outlier for which a dose-dependent curve reaching 10% or less viability was not achievable (Figure 18-19).

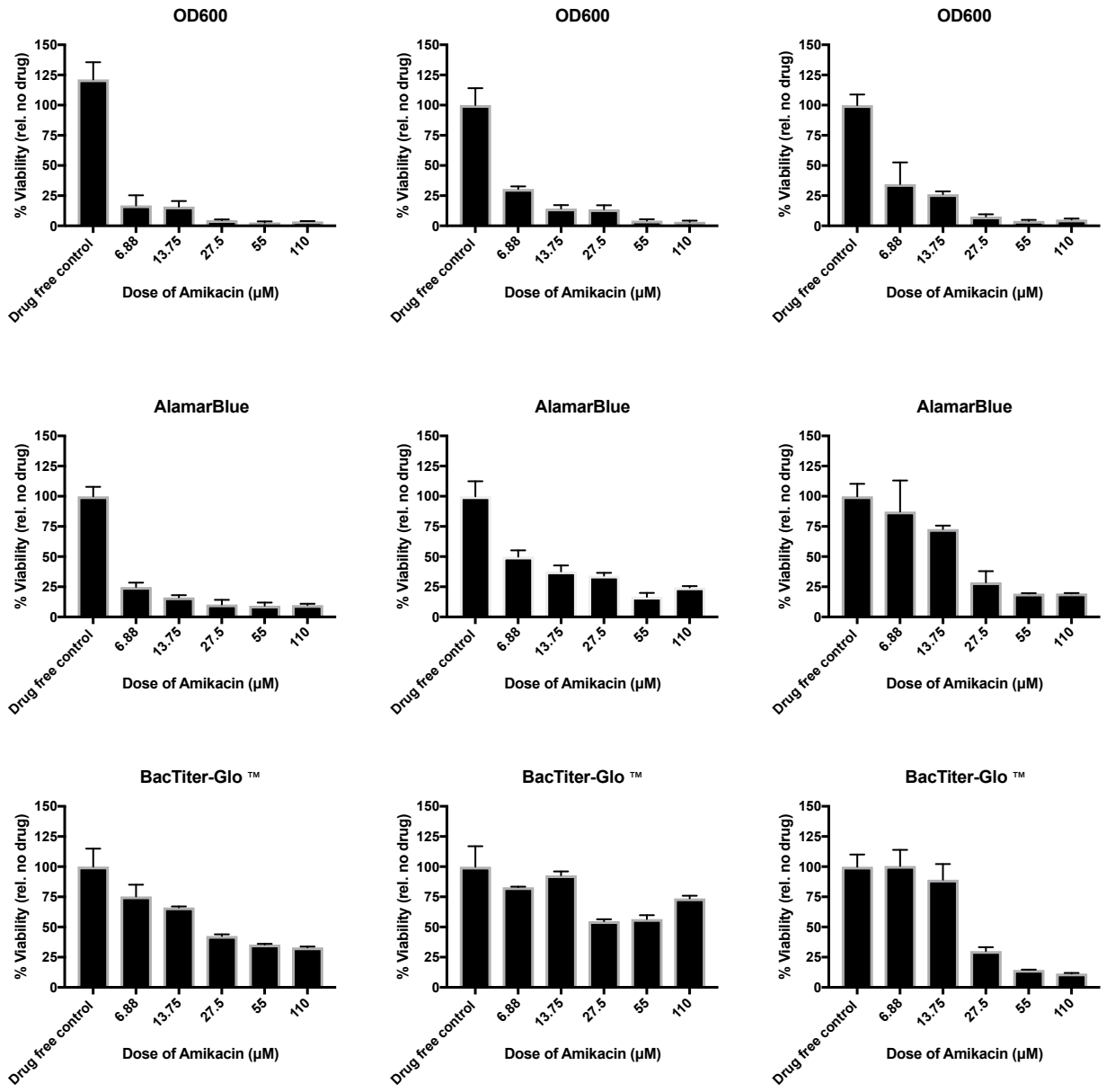

Figure 13. Inter-assay variability was observed for AMK in a reproducible manner. DST was performed for AMK and RIF using OD600, AlamarBlue and BacTiter-Glo thrice. Percent cell viability was assessed, and results are shown as the mean and standard deviation of technical triplicates.

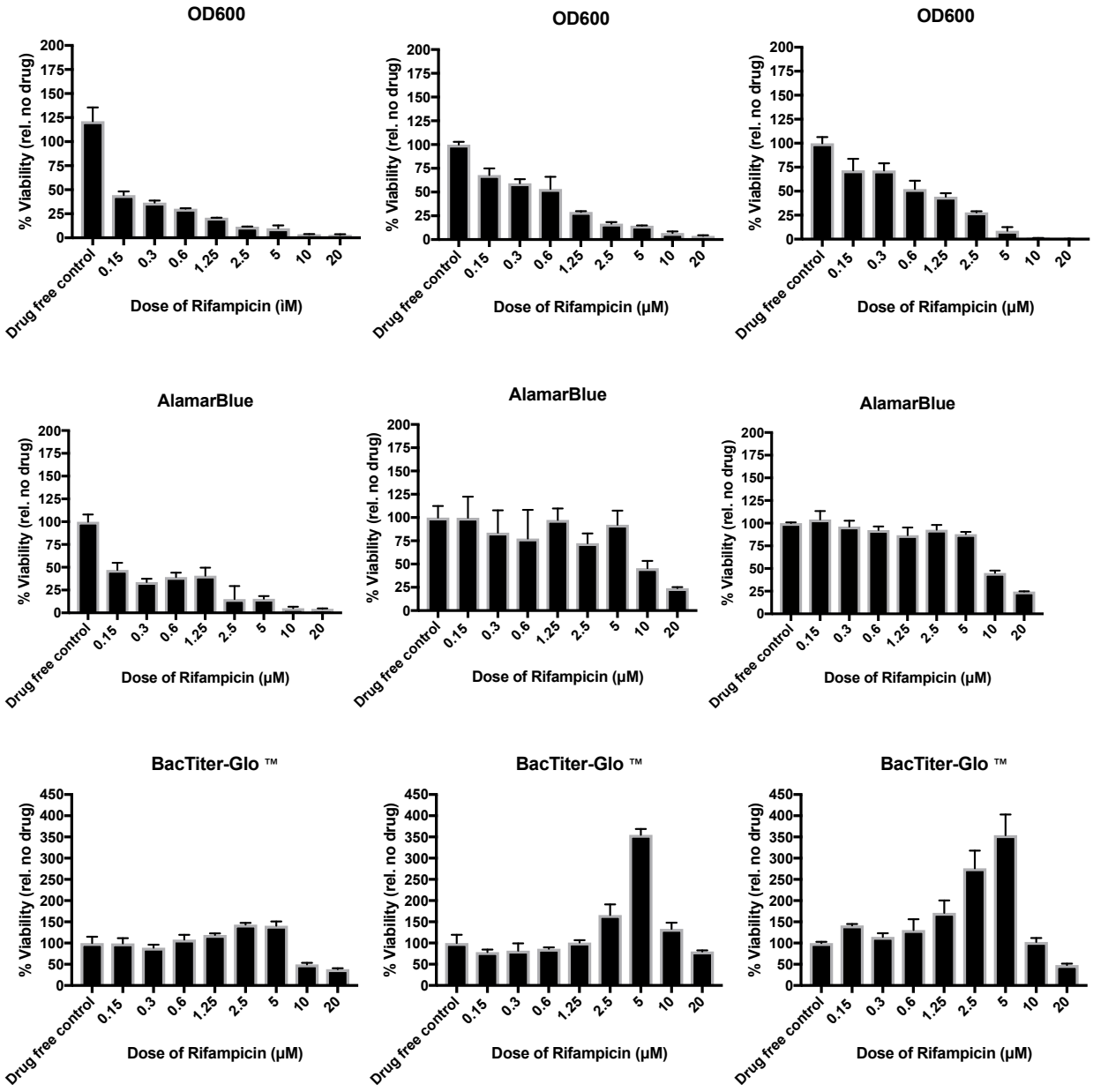

Figure 14. Inter-assay variability was observed for RIF in a reproducible manner. DST was performed for AMK and RIF using OD600, AlamarBlue and BacTiter-Glo thrice. Percent cell viability was assessed, and results are shown as the mean and standard deviation of technical triplicates.

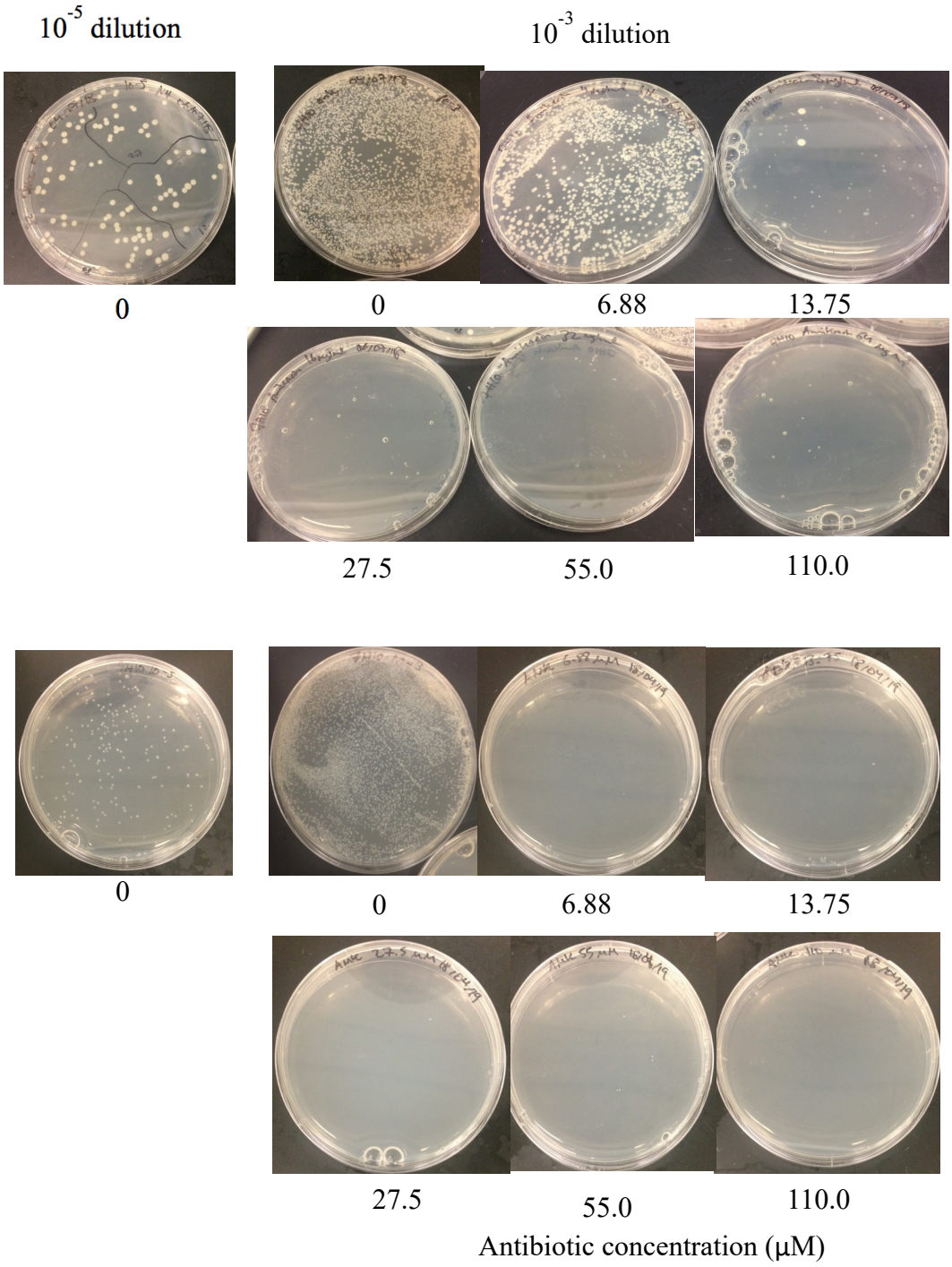

Figure 15. MIC99 was achieved for AMK by agar dilution in a reproducible manner. AMK was tested against *M. abscessus* ATCC 19977 at various doses (0 to 110 µM) in 7H10 agar. Pictures were taken three days after plating.

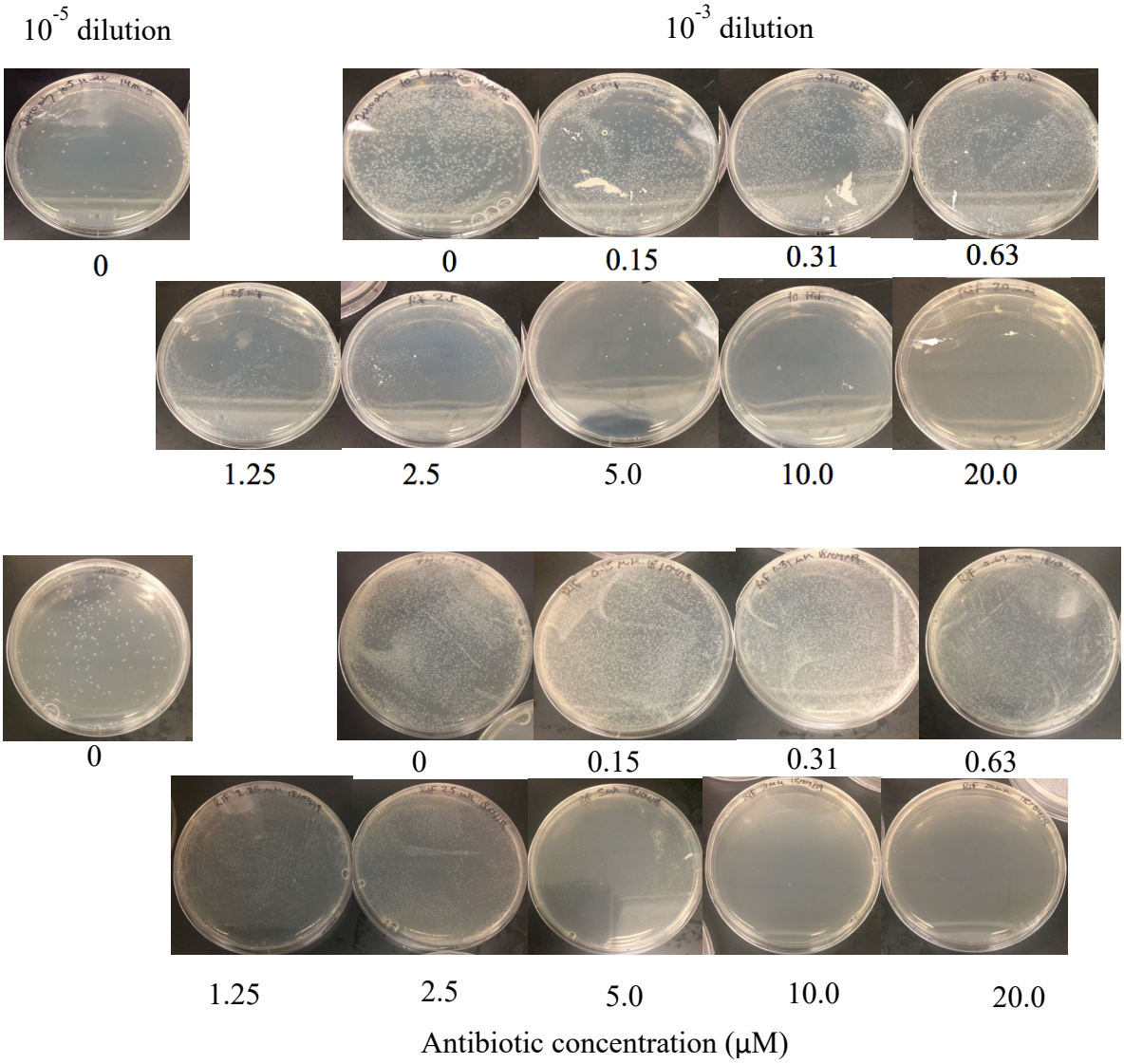

Figure 16. MIC99 was achieved for RIF by agar dilution in a reproducible manner. RIF was tested against *M. abscessus* ATCC 19977 at various doses (0 to 20 µM) in 7H10 agar. Pictures were taken three days after plating.

	AMK			RIF		
	1	2	3	1	2	3
OD600	**MIC90** 27.5 µM	**MIC90** 55 µM	**MIC90** 27.5 µM	**MIC90** 5 µM	**MIC90** 10 µM	**MIC90** 5 µM
AlamarBlue	**MIC90** 27.5 µM	n/a	n/a	**MIC90** 10 µM	n/a	n/a
BacTiter-Glo	n/a	n/a	n/a	n/a	n/a	n/a
Agar dilution	**MIC99** 13.75 µM	**MIC99** 13.75 µM	Not performed	**MIC99** 5 µM	**MIC99** 10 µM	Not performed

Table 3. MIC measured for AMK and RIF using OD600, AlamarBlue, BacTiter-Glo and agar dilution. *n/a indicates that MIC90 was not achieved.

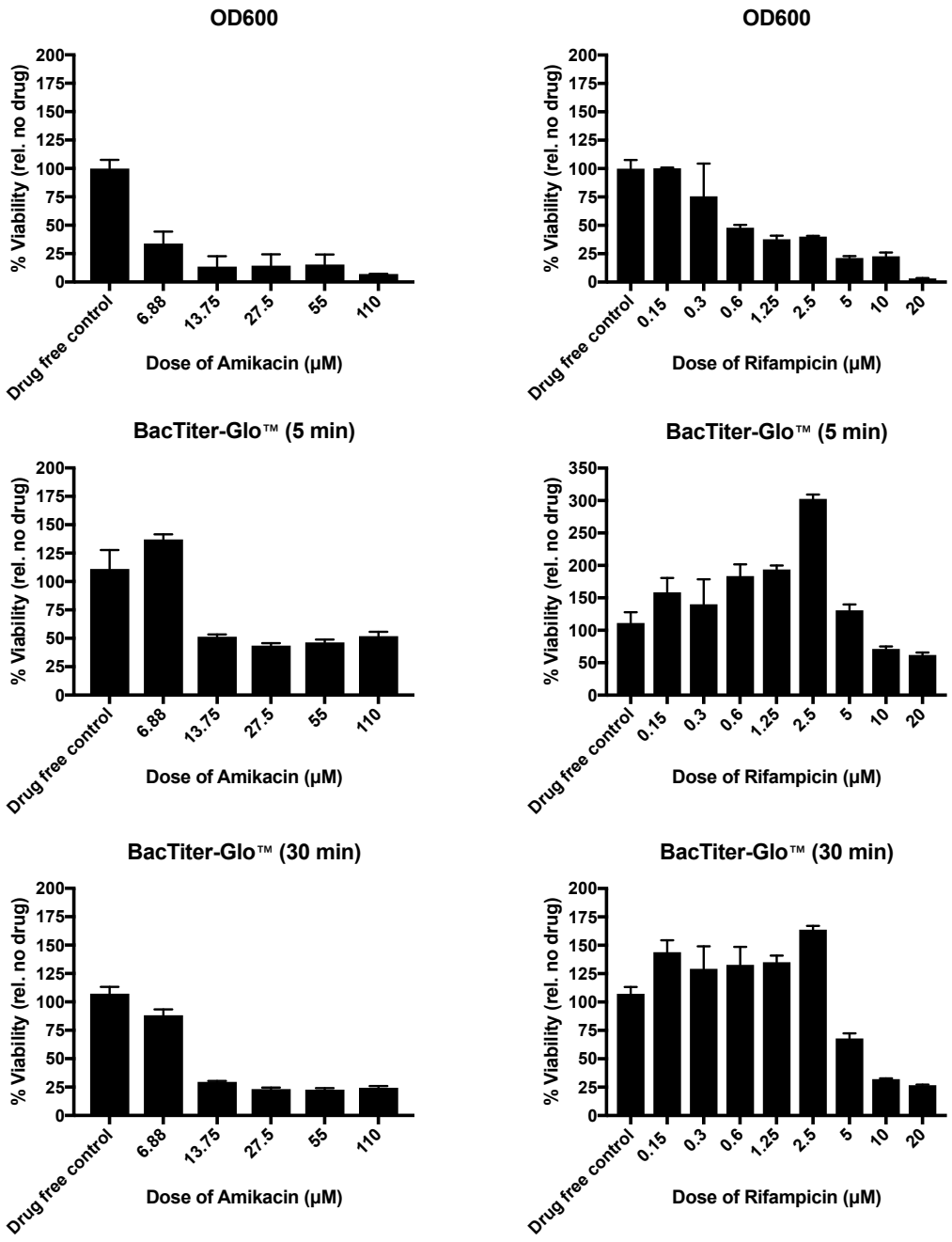

Figure 17. BacTiter-Glo gave discordant results irrespective of 5 or 30 min incubation time. DST was performed for AMK and RIF using OD600 and BacTiter-Glo where different incubation time for the latter were tested, 5 and 30 min respectively. Percent cell viability was assessed, and results are shown as the mean and standard deviation of technical triplicates.

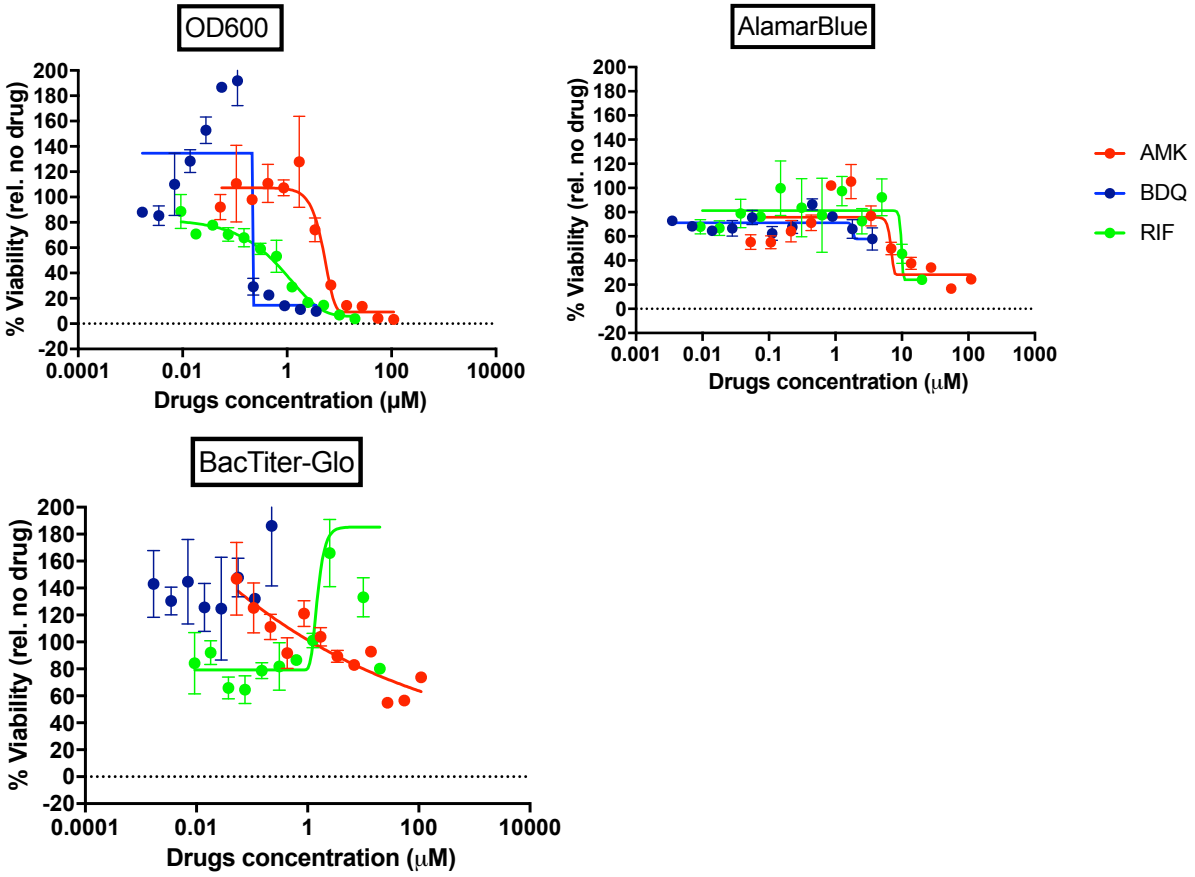

Figure 18. Inter-assay variability was observed for AMK, RIF and BDQ after 3 days of incubation. DST was performed for AMK, RIF and BDQ using OD600, AlamarBlue and BacTiter-Glo. Readings were performed after 3 days of incubation where the data was fitted to the Gompertz equation using GraphPad Prism 7 to calculate MIC99. Percent cell viability was assessed, and results are shown as the mean and standard deviation of technical triplicates.

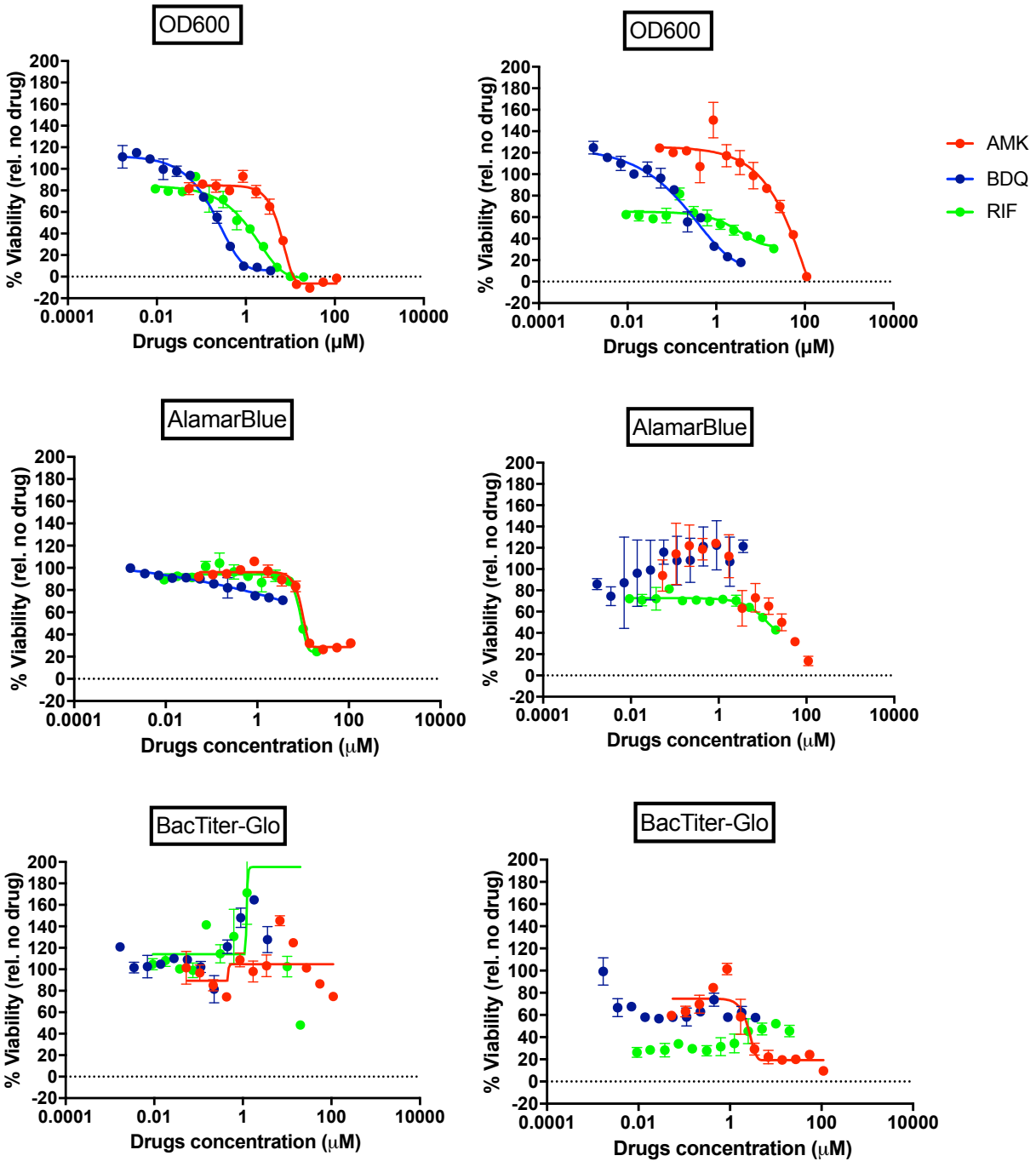

Figure 19. Inter-assay and inter-operator variability were observed for AMK, RIF and BDQ after 2 days of incubation. DST was performed for AMK, RIF and BDQ using OD600, AlamarBlue and BacTiter-Glo by two independent operators in parallel. Readings were performed after 2 days of incubation where the data was fitted to the Gompertz equation using GraphPad Prism 7 to calculate MIC99. Percent cell viability was assessed, and results are shown as the mean and standard deviation of technical triplicates.

		OD600	AlamarBlue	BacTiter-Glo	Agar*
Operator 1	3-day incubation	**AMK** 8.22uM **RIF** 4.69uM **BDQ** 0.23 uM	**AMK** 7.61uM **RIF** 10.44 uM **BDQ** n/a	**AMK** n/a **RIF** n/a **BDQ** n/a	**AMK** 13.75uM **RIF** 10 uM **BDQ** 0.90uM
	2-day incubation	**AMK** 11.93uM **RIF** 6.78 uM **BDQ** 0.83 uM	**AMK** 13.56 uM **RIF** 12.47 uM **BDQ** n/a	**AMK** n/a **RIF** n/a **BDQ** n/a	
Operator 2	2-day incubation	**AMK** n/a **RIF** 10.72uM **BDQ** 2.46uM	**AMK** n/a **RIF** 25.24 uM **BDQ** n/a	**AMK** 3.81 uM **RIF** n/a **BDQ** n/a	**AMK** 55uM **RIF** n/a **BDQ** 0.90uM

Table 4. MIC99 calculated by fitting data to Gompertz equation for AMK, BDQ and RIF.
*Agar plates were incubated for up to 3 days for both operators 1 and 2.
*n/a indicates that MIC99 could not be calculated by GraphPad Prism 7.

4.7. Inter-assay variability trends persist across different operators

In order to examine whether inter-assay variability trends persisted across different operators, the experiment was set up in parallel by two independent individuals. The same initial bacterial culture and initial antibiotic stock of AMK, BDQ and RIF were used by both individuals. The experiments were both set up at the same time and incubated for 2 days at 37°C prior to reading absorbance, fluorescence and luminescence. The general trends observed thus far persisted: OD_{600} and agar dilution showed the highest reproducibility for all three drugs, AlamarBlue showed good reproducibility for AMK and RIF but not for BDQ (Table 4). BacTiter-Glo showed the lowest reproducibility, where >10% viability was only achieved once at the highest dose of AMK but not for RIF or BDQ (contrary to the other broth assays). Inter-operator variability was also observed across assays and tested drugs (Figure 19; Table 4).

5. Discussion

In this study, we set out to evaluate DHFR as a novel drug target in NTM. Herein, we showed how CRISPRi may be used as a rapid and easy method to evaluate gene essentiality. The DHFR target was shown to be essential for *in vitro* growth of *M. smegmatis* and MAB. In order to induce the CRISPRi system for gene knockdown in MAB, a higher dose of ATC was needed compared to *M. smegmatis*. It has been previously shown that MAB contains a tetracycline-modifying monooxygenase [75]. Given that ATC is a derivative of tetracycline, we hypothesize that 0.1 μg/ml ATC is insufficient to induce CRISPRi in MAB due to a hydroxyl group being added to its structure, interfering with target engagement.

The DHFR gene is essential in MAB and targeting this pathway showed promise at achieving growth inhibition. Upon screening our series of compounds using BacTiter-Glo, two inhibitors showed MIC90 values of 5 and 10 μM respectively. However, compounds showing poor or absence of antimicrobial effect against MAB did achieve over 90% growth inhibition against *M. avium* using the same assay, suggesting the potential of DHFR as a drug target for other clinically relevant NTM species. Upon screening the DHFR inhibitors at 10 μM using the OD_{600} assay, we identified, in addition to 1222 and 1245, other compounds that exhibited significant antimicrobial activity against MAB. Indeed, a total of four compounds showed MIC90 values while five additional compounds gave 11-20% viability. At 10 μM, using the OD_{600} assay, SDDC 1222 and 1245 showed approximately 3% and 18% viability respectively. Poor antimicrobial activity against MAB for a few of the SDDC compounds tested may be attributable to the impermeable cell membrane or presence of efflux pumps preventing intracellularly accumulation of the drug. We considered MIC90 as an acceptable initial screening bar for compound selection given that future optimization with respect to the compound's binding ability and cell wall permeability would be possible by our collaborator to increase potency. Overall, our findings suggest that DHFR target would be suitable for drug discovery against MAB.

In drug discovery, the choice of DST method is crucial. Herein, we found that different assays may lead to discrepant results. This was observed with both our novel anti-DHFR compounds as well as known anti-TB drugs AMK, RIF and BDQ. Interestingly, although

BacTiter-Glo appeared to be our most stringent assay to achieve MIC90 for AMK, RIF and BDQ, it was one of the two assays where we achieved MIC90 values for our DHFR compounds. It is worth noting that we screened our compounds with the BacTiter-Glo assay first as we wanted to cross-validate findings from other research laboratories that have been working with the SDDC using this assay. That being said, we showed in this study that cross-validating results with other assays is important. Additional DHFR inhibitors with potent activity against MAB were identified using the OD_{600} assay, and such promising compounds would have been discarded had we relied solely on BacTiter-Glo for our preliminary drug screening.

The assays tested in this study each have its pros and cons. The agar dilution method has long-time been considered the gold standard in DST for bacteria as it allows direct measure of cell viability in a simple and straightforward manner. Pure colonies are obtained which can be used for DNA extraction followed by sequencing to identify mutations of acquired resistance, if desired. The visualization of pure colonies also allows one to ensure through colony morphology that no contamination has occurred. Compared to broth microdilution in a 96-well plate, the risk of cross-contamination is significantly lower. That being said, given the longer turnaround time for results and the labour-intensity involved in large-scale DST testing, agar dilution has progressively been replaced by broth microdilution in many laboratories[55]. Moreover, agar dilution can become more costly than broth DST in a 96-well plate due to more drug being used. There are also concerns of poor diffusion of the drug in the solid media, compound instability in agar over time (due to higher number of incubation days to get results) and inhibition of drug activity by an ingredient in the medium, leading to higher MIC values[76]. For example, certain commercially prepared media are supplemented with thymidine such that the antimicrobial effect of trimethoprim is bypassed and DST results on such media are unreliable[77].

Broth microdilution can generate results within 2-3 days (depending on the NTM species) while allowing large-scale testing of many different conditions (different drugs, strains, doses) in a single 96-well plate. All three broth methods tested in this study are indirect measures of cell viability, however OD_{600} has the advantage of allowing the user to monitor growth with the naked eye. Commercially available microbial cell viability reagents may be preferred due to high sensitivity, 50 cells (AlamarBlue) and 10 cells (BacTiter-Glo) according to the manufacturer's

technical notes. Moreover, previous studies report the limitations of using turbidity readings as a tool to measure MIC for mycobacteria due to cell aggregation, or clumping, leading to an uneven distribution of bacterial cell suspension[27]. However, we found that OD_{600} showed good reproducibility, consistently agreed with the agar dilution method, and was overall more reliable than AlamarBlue and BacTiter-Glo. While inhibition of growth was observed across all tested broth assays for all three drugs, we noticed that 90% inhibition (or more) was achieved at the highest doses tested of AMK, RIF and BDQ consistently with OD_{600} and inconsistently with AlamarBlue and BacTiter-Glo. In many instances, MIC99 could not be calculated from fitting the bacterial viability data with the Gompertz equation for the two latter assays. We also found that depending on the drug itself, different assays may be suitable for DST where interestingly MIC90 was achieved for BDQ with agar dilution and OD_{600} but not with AlamarBlue and BacTiter-Glo. Agar dilution and OD_{600} appeared to be useful for MIC determination for AMK, RIF and BDQ; AlamarBlue for AMK and RIF; and BacTiter-Glo persisted as an outlier. Throughout this study, BacTiter-Glo overestimated percent viability and was in disagreement with OD_{600} and AlamarBlue findings irrespective of the reagent incubation time (5 min vs. 30 min), treatment time (2 days vs. 3 days) and operator.

Different reasons may explain the discrepancy in the results. The AlamarBlue reagent contains blue-colored, non-fluorescent and cell-permeable resazurin which converts into pink, highly fluorescent, resorufin under reducing conditions[78]. As such, cell viability is inferred indirectly from the metabolic activity by measuring the resulting fluorescence intensity. The luciferase-based BacTiter-Glo reagent when added to a growing culture induces cell lysis. Cellular ATP is released, and a luminescent signal is generated. The level of luminescence is proportional to the amount of ATP present, which in turn is proportional to the number of viable cells[79, 80]. Different drugs through their mechanism of action may indirectly or directly alter the pool of secondary metabolites (e.g. ATP and NADPH) in a microorganism. This would affect the readout of indirect cell viability assays that rely on cell metabolism to infer growth, rendering it inaccurate. This could explain the overestimate of percent cell viability observed with AlamarBlue and particularly BacTiter-Glo compared to OD_{600}. To test this theory, we included BDQ as a positive control for BacTiter-Glo as it targets ATP synthase and would directly affect the pool of intracellular ATP[81]. Surprisingly, we found that the assay still yielded high percent

viability at the highest doses tested. Previously, it has been reported that during initial exposure to BDQ, MTB activates ATP-generating pathways to counteract BDQ-induced ATP depletion to enable transient survival[82]. Transcriptional profiling revealed transient induction of the dormancy regulon which triggers a metabolic state that requires less ATP in order to sustain levels in response to stress. This may explain the phenomenon we observed with BacTiter-Glo where although viability decreases at higher drug doses, the percent viability remains higher than that observed with OD_{600}, where MIC90 (or MIC99) is not achieved. MAB may alter its metabolism in response to the stress induced by drug exposure. Hence, microbial cell viability assays such as AlamarBlue and BacTiter-Glo may not suitable to measure specific MICs but rather can be used to get a general estimate of viability. Alternatively, it may also be that the assay is detecting residual ATP released by cell lysis upon drug exposure, separate from cell lysis induced by the addition of BacTiter-Glo. The half-life of ATP was reportedly to be 10 hours in *M. leprae*[83]. Finally, discrepant results were found for DHFR inhibitors between BacTiter-Glo and AlamarBlue. Given that the DHFR reaction is NADPH dependent it may be that targeting this pathway leads to concentrated amounts of NADPH present in the cell that can be used to reduce AlamarBlue more readily[59].

It is expected that MIC values may fluctuate by one twofold dilution above or below the reported critical concentration. However, in certain instances in this study we observed MIC values differing far more extensively than that. There are various sources of variation in the laboratory that may drive this overall variability. As previously discussed, drug stability, the actual parameter measured to infer growth (direct vs. indirect) and strain-to-strain variation, as observed between the laboratory and clinical strains of MAB, can affect results. Upon repeated testing, we found that the extent of MIC drift differed between assays, where agar dilution and OD_{600} were highly reproducible while BacTiter-Glo showed poorest reproducibility overall. In this study, we attempted to control for a few possible sources of variation where both operators used the same initial culture of bacteria and initial antibiotic suspensions of RIF, AMK and BDQ to set up their experiments, in parallel. Interestingly, inter-operator variability still occurred. This may be due to a difference in the starting density of bacteria upon diluting the culture, evaporation of media from the 96-well plate and technical errors. Pipetting errors while serially diluting the drugs can generate a wrong dose and may lead to cross-contamination.

There are few studies discussing inter-assay variability for NTM. Poor correlation between turbidity-based broth microdilution and disk diffusion methods (61% agreement) has been reported for AMK in MAB [84]. One study examined inter- and intra-assay reproducibility of AlamarBlue in MTB for several drugs including RIF. They found that reproducibility depended on the drug under consideration where RIF showed lower levels of variability compared to the other drugs tested, yet a moderate concordance in results overall upon repeated testing [85]. However, they found that it did not affect susceptible-resistant assignment and concluded that the assay's results may be most useful to report drug susceptibility or resistance rather than measuring an exact MIC value. Previous studies have reported the use of AlamarBlue in 7H9 as an acceptable method for MIC determination for *M. avium*[86, 87] However, the use of Resazurin Microtiter Assay (REMA) in 7H9 broth which functions similarly to AlamarBlue, was shown to be less reproducible compared to broth microdilution in Mueller-Hinton medium [88].

The choice of assay to perform DST may depend on the purpose of the study. If the goal is to screen a library of candidate compounds where further optimization of the drug structure is possible, then a sensitive assay would be most appropriate to avoid discarding potentially promising compounds. Together, our findings suggest that OD_{600} is ideal for drug screening due to its ease, reliability and reproducibility. Indirect measures of microbial cell viability using assays that rely on metabolism may not be appropriate. If the goal is to instead identify a lead compound with the highest antimicrobial effect where further optimization will not be possible, then cross-validating results with different assays may be useful.

There is an urgent need for NTM drug discovery to improve treatment outcome. Herein, we showed that DHFR is a novel drug target in MAB based on CRISPRi-mediated gene knockdown and drug screening *in vitro*. A reliable method for MIC measurement is needed to guide compound selection. We showed that different assays yield discordant results and that the choice of assay may depend on the tested drug. Further studies examining inter-assay variability would be needed to elucidate the role of DST in guiding NTM therapy and to manage clinical cases appropriately.

6. Author contributions and funding

M. A. B. conceived the study. N. H. and M. A. B. set up the experimental design for the manuscript. Design and preparation of CRISPRi plasmids was performed by N. H. (for *M. smegmatis* and *M. abscessus*) and M. G. (for *M. smegmatis*). N. H. performed electroporation, RNA extraction and confirmation of gene knockdown by qRT-PCR. C. W. and D. S. provided the DHFR inhibitors. N. H. performed dose-response experiments of the SDDC compounds by broth and agar dilution. N. H. and J. S. performed inter-assay variability experiments of AMK, RIF and BDQ. Generated experimental data was analyzed by N. H. and M. B.

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CHAPTER III
REFLECTIONS AND FUTURE DIRECTIONS

In order to facilitate NTM drug discovery several knowledge gaps with respect to NTM drug targets, resistance and DST methodology need to be addressed. To date, attempts at repurposing currently available TB drugs against NTM have been unsuccessful. We believe that the issue is not necessarily the target but rather within the chemistry itself. Validated anti-TB targets have the potential to leverage NTM drug discovery as long as the newly synthesized compounds are optimized to meet the various NTM-specific challenges related to drug penetration, intracellular accumulation and drug-target binding.

In fact, narrow-spectrum antibiotics tailored to NTM physiology would arguably be most appropriate for NTM treatment given the contrasting drug susceptibility patterns observed at the species and strain levels[41]. Many of the current broad-spectrum antibiotics are ineffective due to intrinsic mechanisms of resistance or possibly genetic redundancy that rescues the phenotype and bypass the antimicrobial effect[5]. Importantly, some of these drugs may also lack the lipophilic propriety that is essential for antimycobacterial drugs to penetrate through the thick cell membrane[89]. Compounds should ideally not carry a large net charge as passive diffusion (as opposed to being actively transported) into the cell would be possible[59]. Alternatively, some have argued that poor correlation between *in vitro* and *in vivo* outcomes can be partially attributed to a lack of bactericidal drugs[90]. It is argued that stationary growth arrest is not sufficient and bactericidal effects would be more promising at reducing re-occurrence of infection.

Furthermore, it is to be noted that our work focused on the overarching idea that compounds against essential genes would be promising for drug discovery. However, this notion has been challenged in the scientific community where it is argued that inhibitors of single essential genes have not yielded successful results over the last few years, and that multi-target agents are showing more promise[91]. In response to this criticism, we would like to highlight that multiple target engagement is achievable by combination therapy and that novel drugs tailored to NTM physiology would still be useful as it can be added to the pool of antibiotics available for multidrug regimens. We believe that combination therapy may remain the safest,

low-risk, strategy to treat NTM patients by delaying the emergence of drug resistance. Combination therapy is perhaps still safer than monotherapy with a multi-target agent as a single gene mutation in the latter may be sufficient to render it incapable of acting against its multiple downstream targets (e.g. a mutation conferring its effluxing or downregulated expression). This would lead to resistance and treatment failure. Alternatively, in multidrug regimens resistance against one agent would in theory not affect the partnering drug's ability to act against its respective target. As such, synergistic studies may be most appropriate to predict clinical outcomes in NTM patients. Hence, much further in the drug development pipeline it would crucial to test the DHFR inhibitors in conjunction with other drugs to assess whether potent inhibitory activity is achievable at lower dosages. This may lower host toxicity and increase patient's adherence to the drug regimen.

In the second half of this study, we demonstrated how inter-assay variability becomes a barrier to drug development. However, this notion is not specific to the field of NTM. In fact, discrepancies in DST results have also been reported for TB where clear data regarding the relationship between genotype, phenotype and response to treatment are lacking. Notable examples include discrepancies between phenotypic and genotypic DST for the detection of RIF resistance[92]. This can lead to delayed or inappropriate treatment and a barrier to optimal patient management. False positives may occur from the inability to discriminate synonymous from nonsynonymous mutations in the rpoB gene. Mutations outside of the region targeted by the molecular assay (e.g. rpoB I491F mutation for the XpertMTB/RIF) are also problematic as this leads to false negative results[92-94].

The professional pathogen MTB has been, and continues to be, studied more extensively in comparison to NTM. Knowledge of MTB drug's mechanism of action and resistance may help elucidate some of the knowledge gaps we currently face with NTM. However, it is important to note that due to the difference in natural reservoir (human vs. environment) both have evolved quite differently overtime. This explains in large part the difference in antibiotic susceptibility we observe where NTM have developed a plethora of resistance mechanisms in order to survive and thrive in its environment[3]. Hence, it may be worth investigating similarities between NTM and other environmental opportunistic pathogens, that are non-

mycobacterial, such as *P. aeruginosa* where both can establish biofilms in the lungs of CF patients[95, 96]. There is already evidence of lateral gene transfer of resistance-conferring genes between MAB and *P. aeruginosa*, where co-morbidity of both bacterial infections in CF patients is common[53].

Finally, as future directions, we hope to potentiate the activity of our DHFR inhibitors through the use of an efflux pump inhibitor. We are interested in combining the compounds with the inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) as it targets the MmpL5/MmpS5 pump system in MTB and orthologs of these pumps have been previously identified in MAB[51, 97]. We are also interested in using a novel bioluminescence resonance energy transfer (NanoBRET) technology with our collaborators at Promega to evaluate the compound's ability to enter the bacterial cell and achieve target engagement. We are currently in the process of optimizing this tool and of co-developing new fluorescent chemical probes to achieve this goal. Once candidate drugs with the lowest MIC are identified, we intend on testing them in a macrophage-like cell line (THP-1 cells) infected with luciferase-producing MAB strain, to verify that antimicrobial activity is maintained intracellularly. For now, the knowledge of the drug's target DHFR provides us a starting place for hit optimization of our most interesting compounds SDDC 1222 and SDDC 1245. Structure-guided drug discovery by X-ray crystallography allows visualization of drug-ligand interactions[98]. A drug's affinity for its target can be inferred and the drug's structure can be altered to enhance binding and promote sustained target occupancy. The structure can also be altered to improve cell permeability should we encounter this as a potential mechanism of intrinsic resistance. Finally, knowledge of the target would also provide us with a starting place to identify mutations conferring acquired resistance.

In the last decades, NTM have emerged as opportunistic pathogens capable of causing chronic, TB-like lung disease. MAB has been deemed an antibiotic nightmare by clinicians due to high-level resistance to most antimycobacterial and broad-spectrum antibiotics. Hydrophobicity of NTM cells plays a major role in chemistry failure against NTM and makes DST for NTM challenging. We would like to conclude this study with the following take-home messages:

(1) NTM drug resistance is most likely due to an issue related to the chemistry and not the drug target; validated TB drug targets may be repurposed for NTM drug discovery.
(2) Reliable DST methodology is crucial to guide compound selection; OD_{600} may be most appropriate for initial drug screening.

Finally, we hope that this study will inform future research efforts for NTM drug discovery and development to improve patient outcome.

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