Novel synergies and isolate specificities in the drug interactions landscape of

Mycobacterium abscessus

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ABSTRACT

Mycobacterium abscessus infections are difficult to treat and are often considered untreatable without tissue resection. Due to the intrinsic drug-resistant nature of the bacteria, combination therapy of three or more antibiotics is recommended. A major challenge in treating *M. abscessus* infections is the absence of a universal combination therapy with satisfying clinical success rates, leaving clinicians to treat infections using antibiotic lacking efficacy data. We systematically measured drug combinations in *M. abscessus* to establish a resource of drug interaction data and identify patterns of synergy to help design optimized combination therapies. We measured approximately 230 pairwise drug interactions among 22 antibiotics and identified 71 synergistic pairs, 54 antagonistic pairs, and four potentiator-antibiotics not previously reported. We found that commonly used drug combinations in the clinic, such as azithromycin and amikacin, are antagonistic in lab reference strain ATCC19977, whereas novel combinations, such as azithromycin and rifampicin, are synergistic. Another challenge in developing universally effective multidrug therapies for *M. abscessus* is the significant variation in drug response between isolates. We measured drug interactions in a focused set of 36 drug pairs across a small panel of clinical isolates with rough and smooth morphotypes. We observed highly strain-dependent drug interactions that cannot be predicted from single-drug susceptibility profiles or known drug mechanisms of action. Our study demonstrates the immense potential to identify synergistic drug combinations in the vast drug combination space and emphasizes the importance of strain-specific combination measurements for designing improved therapeutic interventions.

INTRODUCTION

Mycobacterium abscessus (M. abscessus) is a rapidly growing nontuberculous mycobacterium (NTM) that consists of three subspecies, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (1). *M. abscessus* is a ubiquitous, opportunistic pathogen commonly found in soil, water systems, and contaminated material in hospitals. Recently, a steady increase in the morbidity and mortality of NTM infections has been reported worldwide (2). Although *M. abscessus* causes both pulmonary and extrapulmonary infection, the majority of the clinical syndrome of *M. abscessus* infections are pulmonary, occurring in immunocompromised people and those with pre-existing conditions such as cystic fibrosis, chronic obstructive pulmonary diseases, and bronchiectasis (3, 4).

Pulmonary *M. abscessus* infections are notoriously hard to treat due to the plethora of
intrinsic mechanisms conferring resistance toward most clinically relevant antimicrobials,
including macrolides, aminoglycosides, tetracyclines, and ß-lactams (5). Antibiotics such as
amikacin and moxifloxacin are bactericidal in *E. coli* but are bacteriostatic in *M. abscessus*,
creating another barrier to treating *M. abscessus* infection (6). The inherent phenotypic drug
resistance in *M. abscessus* is due to multiple factors. *M. abscessus* possesses complex drug
efflux pump system. a cell wall rich in lipids and mycolic acids that act as a physical barrier
between antibiotics and the bacteria (7). Additionally, *M. abscessus* express numerous
enzymes that can modify drugs or their targets. For example, studies have identified multiple
enzymes in *M. abscessus* such as the Erm(41) erythromycin ribosomal methyltransferase,
aminoglycoside acetyltransferases, an aminoglycoside phosphotransferase, a rifamycin ADP-
ribosyltransferase (Arr_{Mab}), a ß-lactamase (Bla_{mab}), and tetracycline-modifying
monooxygenase that confer their ability to resist many clinically used antibiotics (7-9). In
addition to intrinsic resistance, acquired mutational resistance against macrolides and
aminoglycosides also poses a significant risk for chronically infected patients with increased
long-term antibiotic exposure (10). These characteristics contribute to the complex and
multifaceted resistome of *M. abscessus*, creating a major challenge in developing
antimicrobial therapies against *M. abscessus*.

Despite being an emerging global health threat, pan-effective treatment for NTM lung
diseases has not yet been established and current treatment guideline is based on small studies
and expert opinion (11, 12). Hence, treatment of *M. abscessus* is highly individualized and
typically split into an intensive initial phase of several weeks comprising at least three
antibiotics and a continuation phase of several months. In patients infected with strains
harboring inducible or mutational resistance to macrolides, a macrolide can be included in the
regimen for its immunomodulatory purpose only (12). Current guidelines suggest a minimum
treatment duration of twelve months after culture conversion, although it is also
individualized based on several factors, such as underlying patient conditions and *M.*
abscessus subspecies, which typically have different clinical outcomes (3, 13). Clinical
outcomes are poor in patients with pulmonary disease and those with immunosuppression (2,
14). Meta-analysis done across multiple clinical studies showed that sputum conversion
without relapse was low, and evidence suggests that surgical resection prolongs negative
culture (13, 15, 16). The poor cure rate and the emergence of clinically acquired pan-
macrolide and pan-aminoglycoside resistance suggest the urgent need to develop more

effective therapies for NTM and *M. abscessus* infections (8). Except for clarithromycin,
results of traditional antimicrobial susceptibility testing by the Clinical and Laboratory
Standards Institute (CLSI) are not linked to clinical outcomes (17). Additionally, there are no
published breakpoints for tigecycline, or repurposed MDR-TB drugs like clofazimine or
bedaquiline that are used to treat *M. abscessus*. Because *M. abscessus* treatment requires
multi-drug therapy, systematic interrogation of drug combinations and drug-drug interactions
has the potential to identify synergistic combinations that will form the basis for more
effective therapies.

Several combination studies for *M. abscessus* have been reported using traditional
checkerboard assays. However, conducting large-scale drug combination measurements is
not practical because systematic measurements of drug-dose combinations in the
checkerboard make these assays too resource-intensive. Here, we utilized DiaMOND
(diagonal measurement of n-way drug interaction), a measurement and analysis pipeline
based on an efficient geometric sampling of a traditional checkerboard assay, to significantly
decrease the number of measurements required in checkerboard assays (18). We modified the
DiaMOND assay to measure potentiation effects of drugs that are not active on their own but
may increase the efficacy of other compounds. Because there are few antibiotics that are
active in *M. abscessus*, potentiator screens may be critical to developing combination
therapies in *M. abscessus* and other NTMs. Using medium-throughput measurement, we
generated a systematic, large-scale catalog of drug interactions for *M. abscessus* as a resource
and evaluated drug interaction patterns. Our dataset includes antibiotics commonly used in
the clinic and next-generation antibiotics, such as bedaquiline or benzimidazole SPR719, an
active moiety of a non-fluoroquinolone gyrase inhibitor SPR720. We identified many novel
synergistic drug pairs, suggesting the potential for effective multidrug regimens in the
combination drug space. However, drug interactions were highly variable among different
clinical isolates highlighting the importance of making isolate-specific drug combination
measurement rather than searching for a universal drug combination to treat *M. abscessus*
infection.

RESULTS

Design of systematic drug interaction study in ATCC19977

To generate a systematic dataset of drug interaction profiles comparable to other published
drug interaction studies, we began by measuring drug combination responses in the *M.*

abscessus reference strain ATCC19977, an *M. abscessus* subsp. *abscessus* variant showing

inducible macrolide resistance due to a functional Erm(41) ribosomal methylase (19). We

measured pairwise combination effects among 22 antibiotics drawn from four categories

(Table 1): **(a)** antibiotics currently recommended for *M. abscessus* treatment such as

amikacin, clarithromycin, and azithromycin, **(b)** anti-tuberculosis drugs such as linezolid and

ethambutol, **(c)** drugs in development for *M. abscessus* infection such as bedaquiline and

SPR719, and **(d)** drugs that show no significant effect on their own but can be used to

potentiate other drugs, such as avibactam.

Table 1. Summary of antimycobacterial used in the study and their antimicrobial activity against *M. abscessus* ATCC19977

	antimycobacterial	general mechanism of action	abbreviation	IC$_{50}$ (µg/mL)	IC$_{90}$ (µg/mL)
active drug	**amikacin**	protein synthesis	AMK	6.6	110
	amoxicillin	cell wall	AMX	300	NA
	azithromycin	protein synthesis	AZM	4.3	680
	bedaquiline	respiration	BDQ	0.16	1.3
	cefoxitin	cell wall	FOX	9	28
	cerulenin	cell wall	CER	2.2	7
	clarithromycin	protein synthesis	CLR	0.45	80
	ethambutol	cell wall	EMB	31	1200
	levofloxacin	DNA	LXF	13	200
	linezolid	protein synthesis	LZD	14	260
	moxifloxacin	DNA	MXF	1.5	35
	nitrofuran	DNA	NFT	44	1800
	rifabutin	RNA	RFB	1.8	23
	rifampicin	RNA	RIF	16	2100
	SPR719	DNA	SPR	0.39	NA
	telithromycin	protein synthesis	TEL	10	NA
	thioridazine	respiration	TZ	23	39
	vancomycin	cell wall	VAN	9	250
potentiator	*avibactam*	ß-lactamase inhibitor	AVI	NA	NA
	streptomycin	RNA	STR	NA	NA
	tetracycline	RNA	TET	NA	NA
	verapamil	efflux pump inhibitor	VER	NA	NA

Drug combinations may be constructed from antibiotics that are active on their own and are effective together. We may then search for synergistic drug combinations, e.g., sets of antibiotics that are more effective together than expected based on their single-antibiotic efficacies. Nevertheless, applying this approach systematically to *M. abscessus* poses a challenge due to its inherent drug resistance and the large-scale, resource-intensive, traditional checkerboard assays. To overcome the latter, we used DiaMOND to measure pairwise combination effects more efficiently. DiaMOND is an experimental analysis method approximating the checkerboard assay to measure drug interactions. The first step of DiaMOND is to determine the 50% and 90% growth inhibitory concentrations (IC$_{50}$ and IC$_{90}$, respectively) of every drug to design an equipotent combination dose-response curve. We sampled 20-25 doses to obtain a dose-response curve for every drug. From obtained IC$_{50}$

values, we designed pairwise DiaMOND experiments for equipotent combinations of drug
pairs (diagonal in Fig. 1A). Fractional inhibitory concentrations at 50% growth inhibition
(FIC_{50}) were calculated based on Loewe Additivity and Bliss Independence null models.
Metrics from both models were strongly correlated ($R^2 = 0.75$, Pearson correlation), so we
focused on FICs calculated by Loewe Additivity (Fig. S1). We report $\log_2 FIC$ values, such
that synergies (negative) are balanced in magnitude compared to antagonisms (positive). We
can also visualize synergies and antagonisms by comparing combination dose-response
curves to single drug dose-response curves (Fig. 1C)

FIG 1. Schematic of drug interaction and potentiation measurement with
DiaMOND. (A) DiaMOND single (axes) and pairwise (diagonal) dose-response
sampling using an equipotent mixture of two drugs. The x- and y-axes show the doses
of the single drugs (X and Y) sampled from low to high concentrations, as indicated
by the blue and yellow gradient. The diagonal is the mixture of half of each single
drug at each dose, as shown by the green gradient. (B) Schematic of antibiotic and
antibiotic-potentiator dose-response measurement. A dose-response for the antibiotic
alone (Y; yellow gradient) and with the addition of a constant concentration of the
potentiating agent (Z; red to orange gradient). The antibiotic-potentiator dose-
response is a mixture of half the dose of the potentiating drug (constant) and half of
the non-potentiating drug (increasing amounts in a dose-response). (C) Schematic of
shifts in dose-response curves with synergy or antagonism. The dose-response curve
in the dashed line represents the effect of a single drug. When the single drug is
combined with another drug, the combination curve might shift to the left, indicating
synergy (blue dot), or shift to the right indicating antagonism (red dot).

Another complementary approach is to design combinations that include potentiators, e.g.,
inactive compounds as single agents that increase the efficacy of other drugs. Similarly, an
inactive drug can also decrease the efficacy of other drugs, and we refer to this effect as

attenuation. This approach could be particularly relevant in designing combination therapy against *M. abscessus*, where there is a dearth of potent antibiotics for clinicians to choose from. For example, SPR741, a synthetic polymyxin analog with little effective against gram-negative bacteria as a stand-alone agent, exhibits synergy in combination with other antibiotics (20-22). In *M. abscessus*, verapamil has minimal activity against ATCC19977 but potentiates the activity of bedaquiline (23). Another reason to search for potentiator-antibiotic pairs is the possibility of reducing treatment doses to alleviate side effects. For instance, intravenous amikacin can cause adverse effects, including gastrointestinal distress (e.g. nausea) and serious cases of ototoxicity and nephrotoxicity (24). A partner drug to amikacin that potentiates its activity could lower the treatment dose and reduce adverse effects.

DiaMOND was initially designed to quantify drug interaction between two potent drugs. Therefore, modification is required to quantify drug interaction between an active drug and a potentiator candidate. Here, we defined potentiator candidates as compounds that did not achieve growth inhibition at a clinically achievable concentration in patients without extreme adverse side effects. We have included four potentiator candidates in our study: avibactam, tetracycline, streptomycin, and verapamil (Table 1). Because potentiator candidates are not active as single drugs, we developed a geometrically optimized sampling of the checkerboard assay to measure the effect of the potentiator candidate at a constant dose on the potency of effective (dose-responsive) antibiotics (Fig 1B). We measured two-dose responses: (a) the single-drug dose-response curve [Y] with increasing concentrations and (b) the single-drug dose-response [Y] (in which drug concentration was reduced by half for all doses) combined with a fixed-dose (1.5x reported maximum plasma concentration after 12-24 hours of dosing in humans) of drug [Z] (Fig. 1B) (25). The effect of the potentiator candidate was calculated as a fold change in concentration of the antibiotic to reach a specific level of growth inhibition (IC_{50} or IC_{90}) with the potentiator candidate compared to the drug alone. This fold shift in IC (FsIC) ratio can be interpreted similarly to FIC values: $\log_2$FsICs are negative for potentiator-drug pairs and positive for attenuator-drug pairs. We focused on evaluating fold shifts at 50% and 90% growth inhibition levels ($FsIC_{50}$ and $FsIC_{90}$, respectively). Potentiation and attenuation can be visualized by the shift of combination dose-response curves relative to single-drug dose-response curves resembling synergy and antagonism, respectively (Fig.1C), with the exception that the combination dose-response utilizes a constant dose of the potentiator candidate with an increasing dose level of the antibiotic.

A drug interaction landscape of ATCC19977

To generate a systematic dataset of drug interactions for *M. abscessus*, we measured 153

pairwise combinations of 18 drugs representing five drug classes in the reference strain

ATCC19977 (Fig. 2A). Among these combinations, we report 122 combinations that passed

our quality control metrics. The details of quality control metrics can be found in the method

section. Briefly, passing criteria include metrics such as Z-factor, the quality of the dose-

response curve fitting, and data reproducibility. The most common reason for quality control

failure was variation in potency that compromised the equipotent design of the combination

dose-response curve, leading to poor equipotency score and reproducibility. Interactions were

measured up to nine times; combinations that failed to achieve at least biological duplicates

that passed these criteria were categorized as unmeasurable and reported as N/A (gray boxes

in Fig. 2A) in this dataset. Approximately 20% of the drug interactions were unmeasurable

due to extreme variation in drug combination response, which is consistent with challenges in

measuring drug susceptibility to *M. abscessus* due to lack of reproducibility (26). Nonetheless,

it is important to understand which drug combinations cannot be reliably measured.

To understand how well DiaMOND drug interaction measurements correlate with other

independent studies, we compared our measurements with previously reported results. We

observed a qualitative (synergy vs. antagonism) agreement between drug interactions from

DiaMOND and other studies. For example, bedaquiline has been shown to eliminate the

bactericidal effect of ß-lactam by dampening the overproduction of ATP to toxic levels

normally found upon treatment with these drugs (27). Using DiaMOND, we observed a mild

antagonism (log_2FIC_{50} of 0.25) between bedaquiline and cefoxitin, a ß-lactam (Fig. 2B). The

antagonism can be visually observed by the right shift of the combination dose-response

curve (solid line), compare to the expected combination dose-response curve if ½

concentration of bedaquiline and ½ concentration of cefoxitin were used (not shown in the

figure). Similarly, we observed a mild antagonism between linezolid and moxifloxacin in

agreement interactions reported in Zhang et al. (log_2FIC_{50} of 0.67 and 0.17, respectively, Fig.

2C) (28). Combinations such as rifabutin and clarithromycin were reported to be synergistic

by Pyrjma et al. (29). Using DiaMOND, we also observed that rifabutin and clarithromycin

are synergistic (log_2FIC_{50} of -0.76 and -3.1, respectively). Taken together, we conclude that

DiaMOND measurements of *M. abscessus* drug interactions are comparable to traditional

checkerboard approaches previously reported in other studies.

In other bacterial species, including *M. tuberculosis* and *E. coli,* drug interactions tend toward

antagonism (30). In our study, we observed a tendency toward synergy in the landscape of

drug interactions in *M. abscessus*. Of the 122 measurable pairwise drug interactions among

18 drugs, about two-thirds (71) were synergistic or additive (with ~ 0 and negative $\log_2FIC_{50}$

values). Our data suggests that there is potential to improve *M. abscessus* multi-drug

regimens using combinations of existing antibiotics. To further explore these combinations,

we analyzed our dataset in smaller sections for interpretability.

FIG 2. Drug interaction landscape of *M. abscessus strain* ATCC19977. (A)
Heatmap of pairwise drug interactions among 18 drugs. Drugs are organized by the
mechanism of action, and drugs recommended for treating *M. abscessus* infection are
indicated in bold text. Drug interactions are evaluated with $\log_2FIC_{50}$ values: $\log_2FIC$
< 0 (synergy, blue) and $\log_2FIC > 0$ (antagonism, red). Gray boxes indicate
unmeasurable drug combinations due to poor reproducibility. Outlined squares
indicate combinations that are shown in the dose responses below. (B-E) Example
pairwise (and corresponding single-drug) dose responses. Pairwise dose responses for
synergistic and antagonistic combinations are shifted to the left and right, respectively,
as compared to the corresponding single-drug dose-response curves. Circles with gray
indicate the IC_{50} of single drugs, and circles with the red-blue scale according to
$\log_2FIC_{50}$ values as in (A). The y-axis represents growth inhibition, whereas the x-axis
represents the dose unit (a unitless representation of the volume, or concentration,
used for each drug. A dose unit is preferred when plotting as different drugs have
different inhibition concentrations, while a dose unit normalizes the difference and
allows easy representation) (B-C) Dose-response curves of combinations that were
reported from other studies which are bedaquiline (BDQ) + cefoxitin (FOX) (B) and
linezolid (LZD) + moxifloxacin (MXF) (C). (D) Dose-response curves for clinically
relevant combination amikacin (AMK) + azithromycin (AZM) (antagonistic) and (E)
novel combination amikacin (AMK) + bedaquiline (BDQ) (synergistic).

Amikacin and macrolides (azithromycin and clarithromycin): Combination treatment
with amikacin and azithromycin (or clarithromycin) is a recommended treatment for
macrolide-susceptible *M. abscessus* (12). We observed a strong antagonistic relationship
between amikacin and a commonly used macrolide, azithromycin (Fig. 2D, $\log_2FIC_{50}$ of 1.2).
We also tested another macrolide-amikacin pair (clarithromycin-amikacin) but found that the
combination was unmeasurable because the data fluctuated significantly from replicate-to-
replicate. Generally, we did not observe any strong synergistic combinations between
amikacin and other tested drugs, except with amoxicillin, bedaquiline, and nitrofurantoin
($\log_2FIC_{50}$ of -0.58, -0.43, and -0.36, respectively). None of these drugs are currently being
recommended to be used with amikacin. For azithromycin and clarithromycin, we found
more synergistic combinations between these macrolides and other tested drugs. Both
azithromycin and clarithromycin are strongly synergistic with linezolid ($\log_2FIC_{50}$ of -1.1 and
-0.99, respectively), an oxazolidinone that is suggested to treat *M. abscessus*. Azithromycin

and clarithromycin are also synergistic with cell-wall-acting drugs (except with cerulenin),
RNA polymerase inhibitors, and respiratory inhibitors (Fig. 2A). These data suggest that
there is potential to improve treatment outcomes by combining these core bacteriostatic
protein elongation inhibitors with antibiotics that disrupt initiation of protein synthesis
(linezolid) or target other cellular processes.

Clinically used antibiotics and other common antituberculosis agents: Besides amikacin
and macrolides, other drugs recommended for treating *M. abscessus* are imipenem, cefoxitin,
tigecycline, clofazimine, and linezolid (12). Among this list, we focused on linezolid and
cefoxitin due to drug availability and reproducibility. **Linezolid:** We observed synergies
between linezolid and some of the drugs used in the clinic, such as azithromycin and
clarithromycin ($\log_2 FIC_{50}$ of -1.1 and -1.0, respectively). Linezolid is strongly antagonistic
with DNA-targeting antibiotics in our drug set while synergistic with cell-wall-acting drugs
and respiratory inhibitors (Fig 2A), suggesting an underlying relationship between drug
interaction and mechanism of action. We did not observe a synergistic relationship between
linezolid and amikacin, which was previously reported in another study (28). However, there
are several differences between the two studies that may account for these differences,
including the media used (7H9 medium with supplements vs. CAMHB), strains
(ATCC19977 vs. clinical isolates from patients), and method used to determine drug
interaction (DiaMOND vs. broth microdilution). **Cefoxitin:** Cefoxitin is one of the few ß-
lactams recommended to treat *M. abscessus*, besides imipenem (31). We could not obtain a
reliable dose-response curve for both imipenem and cefoxitin, which may be due to the
instability of ß-lactams in solution or the presence of the bla_{mab} gene that confers their
resistance to ß-lactam. Nevertheless, one combination that passed our quality control is
bedaquiline and cefoxitin ($\log_2 FIC_{50}$ of 0.25). The antagonistic relationship between
bedaquiline and cefoxitin has been reported in another study (32). It is thought that cefoxitin
(and imipenem) triggers an ATP burst in *M. abscessus* by increasing oxidative
phosphorylation, which is suppressed by bedaquiline, an F-ATP synthase inhibitor, leading to
an elimination of the bactericidal effect of cefoxitin against *M. abscessus* (32, 33). Though
this result is preliminary, it suggests that combining ß-lactams with bedaquiline could be
unfavorable. **Moxifloxacin:** Moxifloxacin and other fluoroquinolones have been shown to
have good activities against *M. abscessus* isolates *in vivo* (34). We and others have observed
that moxifloxacin is antagonistic with azithromycin *in vitro* ($\log_2 FIC_{50}$ of 0.23) and *in vivo*
(35). We also observed that moxifloxacin is antagonistic with recommended antibiotics such

as amikacin and linezolid ($\log_2$FIC$_{50}$ of 0.31 and 0.68 respectively), suggesting its inclusion

in multidrug therapies should be carefully considered. **Ethambutol:** Ethambutol is another

antituberculosis agent with potential activity against NTMs such as *Mycobacterium avium*

complex (MAC) due to its ability to slow the acquisition of macrolide-resistance clinically

(36). The interactions of ethambutol with other drugs used to treat NTMs are unknown. Here,

we observed a synergistic relationship of ethambutol with linezolid or azithromycin but not

amikacin ($\log_2$FIC$_{50}$ of -0.79, -0.28 and 0.4 respectively), suggesting potential for exploring

of ethambutol for *M. abscessus* treatment. **Rifampicin:** Rifamycin is a common

antituberculosis agent that is highly potent *in vivo* and reduces relapse rate in combination

therapies (37). However, rifampicin is not commonly used for *M. abscessus* because they are

known to be intrinsically resistant to rifampicin (38). *M. abscessus* resistance to rifampicin

partially due to its intrinsically low cell-wall permeability, possibly due to high lipid content,

as well as drug efflux pump (37, 39). Additionally, *M. abscessus* encodes rifampicin ADP-

ribosyltransferase (Arr$_{Mab}$), whose function is to catalyze ADP-ribosylation and render

rifampicin inactive (40, 41). Though rifampicin does not have high potency against *M.*

abscessus, our dataset demostrate synergistic interaction between rifampicin with many

tested drugs, including amikacin and azithromycin. Rifampicin also synergizes all cell-wall-

acting antibiotics in our dataset; the mechanism of synergy may be due to a weakened cell

wall allowing better entry for rifampicin. Though rifabutin, another rifamycin antibiotic, has

been approved to treat Mycobacterium Avium Complex and TB, its potential against *M.*

abscessus has not been fully explored. Recent findings demonstrate limited modification of

rifabutin by the Arr$_{Mab}$ in comparison to rifampicin, resulting in lower MICs against both the

ATCC19977 strain and clinical *M. abscessus* isolates (42). We also observed in our dataset

that rifabutin has a lower IC$_{50}$ and is more potent than rifampicin (IC$_{50}$ of 1.8µg/mL versus 16

µg/mL, respectively, Table 1, Fig. 4A). However, rifabutin in our pairwise combinations tend

toward antagonism, indicated by more red squares in the heat map compared to rifampicin

(Fig. 2A). Nevertheless, rifabutin remains synergistic with frontline *M. abscessus* antibiotics

such as clarithromycin (which has also been reported by other study), amikacin, and

azithromycin, suggesting that rifabutin may be part of a clinically useful combination therapy

(43). The rifamycin pairwise interaction data support continued exploration of this important

class of drugs for treatment of *M. abscessus* infections.

Non-traditional NTM antibiotics

Next, we evaluated an unexplored space in combination therapies by analyzing drug

interactions among antibacterials that are not traditionally used to treat NTMs and TB.

Telithromycin: Telithromycin belongs to a large group of ketolides, a newer generation of

macrolide with a slightly different mechanism of action (44, 45). Ketolides appear to partially

inhibit protein synthesis rather than complete or near-complete inhibition of protein synthesis,

which leads to more cellular degradation and a stronger bactericidal effect (45, 46). Despite

being designed to be an alternative for some macrolide-resistant bacteria, telithromycin is not

often used in the clinic due to its toxic side effects on the liver (47). We observed particularly

strong synergies between telithromycin and other antibacterial compounds such as

nitrofurantoin, rifampicin, amoxicillin, thioridazine, and azithromycin ($\log_2 FIC_{50}$ of -1.7, -1.7,

-2.7, -2.4, and -0.76, respectively), suggesting the potential of using combinations containing

telithromycin to treat macrolide-resistant *M. abscessus*. Nevertheless, telithromycin is mildly

antagonistic with amikacin and additive with linezolid ($\log_2 FIC_{50}$ of 0.09 and -0.02,

respectively). Therefore, further evaluation may be required before combining telithromycin

with these clinically recommended drugs. **Amoxicillin and vancomycin:** Cell-wall-acting

antibiotics amoxicillin and vancomycin show broad synergies with different classes of

antimycobacterial (Fig. 2A). We found that amoxicillin is synergistic with almost all the

tested partner drugs, including amikacin, azithromycin, clarithromycin, rifampicin, and

ethambutol ($\log_2 FIC_{50}$ of -0.58, -0.94, -0.87, -1.2, and -1.2, respectively). Amoxicillin has

previously been known to enhance the effect of ß-lactam antibiotics such as imipenem and

relebactam (48). Our results suggest that amoxicillin may also partner well with a broad

range of antibiotics. For example, amoxicillin and telithromycin are strongly synergistic, with

a $\log_2 FIC_{50}$ of -2.7 (the most synergistic combo in our dataset). Vancomycin is another

widely synergistic drug in our drug panel and was reported to be synergistic with

clarithromycin, one of the most used clinical drugs for *M. abscessus* (49). Although we did

not obtain a reliable drug interaction measurement for vancomycin with clarithromycin due to

poor reproducibility, vancomycin is a promising candidate for a multi-drug regimen which

showed broad synergy with most of the other drugs tested, except with thioridazine (Fig. 2A).

Together, these data suggest that telithromycin, vancomycin, and amoxicillin, despite being

uncommon drugs to choose in the clinic, tend toward synergy and may be candidates as

partner drugs for *M. abscesses* regimen development.

Bedaquiline

Another drug candidate for the combination treatment of *M. abscessus* is bedaquiline, a

diarylquinoline that inhibits subunit c of mycobacterial ATP synthase. In a zebrafish model of

M. abscessus infection, bedaquiline was shown to exert a therapeutic effect by preventing the

formation of abscesses (50). Additionally, a preliminary study in the clinic demonstrated the

efficacy of using bedaquiline as salvage therapy. Within three months of treatment,

bedaquiline reduced the bacterial load in the sputum of patients (51). Consistent with this

finding, we found bedaquiline to have a low IC_{50} value of 0.16 µg/mL (Table 1). With

DiaMOND, we observe that bedaquiline mildly antagonizes cefoxitin and amoxicillin

(log_2FIC_{50} of 0.25 and 0.14, respectively) and is mildly synergistic with vancomycin and

ethambutol (log_2FIC_{50} of -0.35 and -0.08. respectively). The antagonistic relationship

between bedaquiline and cefoxitin in our data set is in agreement with previous studies that

suggested bedaquiline eliminates the effects of ß-lactams (52). To evaluate the potential of

bedaquiline more fully, we investigated drug interactions between bedaquiline and other

antibiotics where these drug interactions have not yet been reported. We observed an overall

trend that bedaquiline is antagonistic with DNA-acting antibiotics used in this study (Fig. 2A).

In contrast, bedaquiline is synergistic with protein synthesis targeting drugs and thioridazine

(Fig. 2A). Bedaquiline's potency and synergy with clinically favored protein synthesis

inhibitors, such as amikacin and azithromycin (log_2FIC_{50} of -0.42 and -0.35, respectively)

have not been previously reported to our knowledge. However, the administration of

bedaquiline following amikacin yields positive clinical outcomes and has been reported for

macrolide-resistant *M. fortuitum* complex soft tissue infection (53). A small preliminary

report also suggested the potential clinical and microbiologic activity of bedaquiline against

M. abscessus (51). Though current clinical results on bedaquiline are limited and further

studies are required, these results suggested that bedaquiline should be explored for treating

M. abscessus, and combinations containing bedaquiline could be considered for macrolide-

resistant cases.

Thioridazine is an atypical antipsychotic still available in generic form, but with limited

clinical use because of QTc prolongation (54). This agent initially gained interest for its

potential use in treatment of MDR-Tb, as it has been shown to have properties against Mtb *in*

vitro, *in vivo*, and in clinical cases, irrespective of antibiotic resistance status (55). In this

study, thioridazine is synergistic with clinically preferred drugs such as amikacin,

azithromycin, and clarithromycin (log_2FIC_{50} of -0.04, -0.46, and -0.22, respectively), as well

as drugs that are not traditionally used for NTM treatment such as telithromycin, and

bedaquiline ($\log_2FIC_{50}$ of -2.4 and -0.72, respectively). Although thioridazine does not appear

to have a strongly synergistic drug interaction profile (Fig. 2A), it is one of a few drugs in this

dataset that shows synergy with clinically preferred drugs (other drugs are bedaquiline,

vancomycin, and amoxicillin), suggesting that non-traditional antibiotics such as thioridazine

could be useful in combination with other agents for *M. abscessus* infection, where the

intrinsic resistance to common antibiotics is a challenge for treatment success.

SPR719

Another promising candidate for *M. abscessus* multi-drug regimen development is SPR719.

SPR719 is a DNA gyrase inhibitor (GyrB) that is currently in clinical studies as a treatment

for NTM infections, and is the prodrug version of SPR720, which is in phase I clinical

development as a new oral agent for NTM infection and Mtb infection (56). We determined

the IC_{50} of SPR719 to be 0.39 µg/mL (Table 1), in agreement with the reported IC_{50} from

previous studies (0.25 – 4 µg/mL, (26)). Despite being highly potent, the interaction profile

of SPR719 is relatively mild, with a balanced profile of synergies and antagonisms (an even

number of synergistic combinations, having $\log_2FIC_{50} < 0$ and antagonistic combinations

$\log_2FIC_{50} > 0$ (Fig. 2A)). SPR719 is additive with amikacin ($\log_2FIC_{50}$ of -0.09) and

synergistic with drugs from a broad range of classes used to treat NTMs and TB, including

azithromycin, telithromycin, rifampicin ($\log_2FIC_{50}$ of -0.49, -0.26 and -0.19, respectively),

and all cell-wall acting antibiotics tested (Fig. 2A). Through genomic analysis, *M. abscessus*

was shown to contain a natural A92S mutation in *gyrB*, which is unique among other NTMs

and *M. tuberculosis* (57). This mutation causes a conformational change in the ATP binding

site, which may partially explain the intrinsic resistance of NTMs to GyrB inhibitors (57, 58).

Despite this mutation, SPR719 is still very potent against *M. abscessus* (Table 1, Fig. 4A)

(58). SPR719 is more potent than the fluoroquinolone moxifloxacin and is synergistic with

more combinations compared to moxifloxacin (moxifloxacin $\log_2FIC_{50}$ median is 0.22 with

only four combinations having $\log_2FIC_{50} < 0$) (Table 1, Fig. 4A). Although moxifloxacin is

not commonly recommended to treat *M. abscessus*, moxifloxacin is still being used in

conjunction with amikacin and macrolides in clinical settings (12, 59). Together, these data

suggest that SPR719 may be part of improved combination therapies for *M. abscessus*,

including as a replacement for moxifloxacin.

Antibiotic potentiation to improve combinations therapy against *M. abscessus*

To determine whether a potentiator candidate enhances or attenuates the potency of
antibiotics, we measured how the addition of potentiator candidates shifts the partner
antibiotic's dose-response curve (Fig. 1B-C, Fig. 3A). We quantified the degree of
potentiation (and attenuation) by calculating the $\log_2$-transformed fold shift in IC_{50} or IC_{90} of
the effective antibiotic with the addition of a constant dose of the candidate potentiator so that
a negative $\log_2$FsIC indicates potentiation and a positive value indications attenuation (Fig.
1B-C). We observed that all tested antibiotics were attenuated by all four potentiator
candidates at IC_{50}, but some antibiotics were potentiated at IC_{90} (Fig. 3A). For example,
avibactam attenuates the effect of amikacin at IC_{50}, indicated by the right shift in combination
dose-response curves compared to the amikacin dose-response curve ($\log_2$FsIC$_{50}$ of 1.1, Fig.
3B). However, with avibactam, amikacin reached IC_{90} at a lower concentration, indicated by
a left shift in the combination dose-response curve compared to single-drug dose-response
curve ($\log_2$FsIC$_{90}$ of -2.1, Fig. 3B), indicating a potentiating effect. We observe a similar shift
in all combinations where IC_{90} is reported, e.g., potentiator candidates shifted from being
attenuating to either less attenuating (such as bedaquiline with streptomycin; $\log_2$FsIC shifts
from 6.4 at IC_{50} to 4.2 at IC_{90}), or from attenuating to potentiating (as with the previous
example between amikacin and avibactam), or from less potentiating to more potentiating
(such as avibactam and rifabutin; $\log_2$FsIC shift from 0.04 at IC_{50} to -3.4 at IC_{90}, Fig. 3B).
This dramatic shift from attenuation to potentiation is explained by an increase in the
steepness of the dose-response curve with the addition of potentiator candidates so that the
effect in some combinations is an increase in potency at higher dose levels of the antibiotic
(Fig. 3B). Among four tested potentiator candidates, streptomycin has the strongest
attenuating effect at IC_{50} and IC_{90} (except with rifabutin and levofloxacin, in which case
streptomycin acts as a potentiator). Among our tested drug set, three inactive drugs potentiate
the activity of rifabutin (with the 4th being unmeasurable).

FIG 3. Effect of potentiator candidates on antibiotic efficacies in *M. abscessus* ATCC19977. (A) Heat map of pairwise drug interactions with potentiators at a fixed concentration. Drugs are categorized based on their mechanism of action and drugs recommended for treating *M. abscessus* infection are indicated in bold text. Drug interaction measurement is expressed as the change in log2 fold shift at IC_{50} (left) or IC_{90} (right). $Log_2FsIC < 0$ indicates potentiating effects, shown in blue, and $log_2FsIC > 0$ indicate attenuating effects, shown in red. Outline squares indicate combinations that are shown in the dose-response below. (B) Example dose-response curves showing the fold shift in IC_{50} and IC_{90} for drugs combined with potentiators, amikacin (AMK) + avibactam (AVI) (top), bedaquiline (BDQ) + verapamil (VER) (middle), rifabutin (RFB) + avibactam (AVI) (bottom). The dash line indicates the single drug dose-response curve, while the solid line represents the combination curve. A left shift in the solid line dose-response curve indicates a potentiating effect, and a right shift shows an attenuated effect by the potentiator.

Beta-lactams are the most widely used antibiotics (60). However, due to the presence of the

broad-spectrum beta-lactamase Bla_{Mab}, only imipenem and cefoxitin are currently

recommended in the multidrug-regimens targeting *M. abscessus* (8). Therefore, potentiators

such as avibactam, a non-ß-lactam ß-lactamase inhibitor shown to efficiently inhibit Bla_{Mab},

can potentially help extend the spectrum of ß-lactam antibiotics active against *M. abscessus*

(61). Porins of *M. abscessus* cell wall have been shown to partially contribute to ß-lactam

resistance as they allow hydrophilic molecules to cross the cell membrane, which can interact

with the target in the cytoplasm and potentially trigger the expression of resistance genes (10,

62). Avibactam has also been reported to reduce the MIC of cell-wall-acting agents (63), and

we observed a potentiating effect of avibactam with cell-wall-acting agents ethambutol,

cerulenin, and vancomycin (log_2FsIC_{90} of -0.76, -0.072, and -1.9) at IC_{90}. Because avibactam

is a ß-lactamase inhibitor, we also wanted to determine its effect with amoxicillin. Due to the

low potency of amoxicillin, there is no IC_{90} for amoxicillin. However, at IC_{50}, we observed a

mild potentiating effect (log_2FsIC_{50} of -0.2), which is not commonly observed in our dataset

(the other combinations are avibactam and nitrofurantoin, log_2FsIC_{50} of -0.2, avibactam and

rifampicin, log_2FsIC_{50} of -0.44, and avibactam with thioridazine, log_2FsIC_{50} of -0.061).

Although verapamil is not effective on its own and have limited clinical used due to its

primary effect on cardiac function, verapamil at 50 µg/mL was reported to enhance the

killing of bedaquiline in *M. abscessus* (23, 64). In contrast, our data show a right shift

(attenuation) of the bedaquiline + verapamil dose-response curve compared to the

bedaquiline single dose-response curve (log_2FsIC_{50} value of 1.9, Fig. 3B). This difference in

the role of verapamil on bedaquiline efficacy may be due to experimental differences such as

verapamil concentration. In Viljoen et al., 50µg/mL of verapamil was used, whereas we used

a lower dose of 2.35 µg/mL, which was chosen based on 3-fold maximum plasma

concentration 4-6 hours after administration, assuming one pill of 100 mg of bedaquiline was

administered (65).

We observed that streptomycin and verapamil largely attenuated the potency of partner drugs,

whereas avibactam and tetracycline are broadly potentiating at IC_{90} (Fig. 3A), suggesting that

these two candidates should be further explored as an element of multi-drug treatment for *M.*

abscessus infection. Additionally, we showed that with modification, DiaMOND can be used

to measure the combined effect between a potentiator and an active drug.

Drug interactions cannot be predicted from single drug potencies or mechanisms of

action

In the absence of a known effective multidrug therapy for *M. abscessus* infection, clinicians

usually rely on single drug susceptibility profiles and their own experience to determine the

best drug combination for each patient. In some cancers, single-drug susceptibility profiles

can be used to design optimized combination therapies under the principle that efficacy for

each cancer is determined by its susceptibility to any of the agents and that combination

therapies were effective as bet-hedging strategies (66). To understand whether we could

predict drug interactions based on single-drug properties, we started by evaluating whether

single-drug potencies were correlated with the propensity for drug interactions to be

synergistic or antagonistic in *M. abscessus*. In Fig 4A, single drugs are organized based on

their IC_{50} on the x-axis, and the y-axis shows the distribution of log_2FIC_{50} of all combinations

containing that single drug. We do not observe a correlation between distributions of

log_2FIC_{50} compared to IC_{50} for each drug, suggesting that drug interactions cannot be

predicted from single drug potencies. We wondered if the most potent or least potent

antibiotic is the driver of drug interaction, which may be obscured by looking at the overall

propensity for synergy compared to single-drug potencies. To take the IC_{50} of both drugs in

each pairwise combination into consideration, we evaluated whether there were patterns of

synergy and antagonism compared to the IC_{50}s of both partner drugs (Fig. 4B). In the drug

interactions among 18 antibiotics, there was no clear trend of synergy and antagonism based

on single-drug potencies (e.g., the drug interactions, colored by log_2FIC_{50}, are not clustered

by drug potency to either drug). For example, SPR719 and bedaquiline are highly potent

drugs with low IC_{50} (IC_{50} of 0.39 µg/mL and 0.16 µg/mL, respectively), but the combination

between SPR719 and bedaquiline are antagonistic (log_2FIC_{50} of 0.74). Conversely, amikacin

and amoxicillin are not as potent as SPR719 or bedaquiline (IC_{50} of 6.6 µg/mL and 300

µg/mL, respectively) but are mildly synergistic in combination (log_2FIC_{50} of -0.59). Together,

our analysis demonstrates that single drug potency is not predictive of drug interactions, and

we cannot anticipate whether a drug pair will be synergistic or antagonistic based on their

potency profiles as single agents.

FIG 4. Relationship between drug potency or mechanism of action with synergy

(A) Box plot of drug interaction ($\log_2 FIC_{50}$) of all combinations containing the single

drug represented on the x-axis, drugs recommended for treating *M. abscessus*

infection are indicated in bold text. Each dot inside the box represents a combination

of that single drug and another drug in the data set. Single drugs are ordered on the x-

axis based on IC_{50} (Table 1), with drugs having the highest IC_{50} on the right and the

lowest IC_{50} on the left (B) Comparison of drug interaction scores with single drug

potency in ATCC1977. Pairwise combinations are plotted as the components of their

single drug IC_{50} (μg/mL). Given a pairwise combination, the potency of a single drug

is plotted on the y-axis while the non-potent drug is on the x-axis; drugs

recommended for treating *M. abscessus* infection are indicated in bold text. The color

fill indicates the synergistic (blue) or antagonistic (red) interaction in ATCC19977. (C)

Clustering of drugs' interaction profile in ATCC19977. Each box represents the

Pearson correlation of drug interaction profiles between two antibiotics. 1 indicates a

positive correlation, whereas -1 indicates a negative correlation. The color bar along

the y-axis represents the general drug target pathway (e.g., inhibition of protein, cell

wall, DNA, or RNA synthesis and inhibition of respiration.)

Next, we evaluated whether there were drug interaction patterns that correspond to each

partner drug's mechanism of action. To test the hypothesis that drugs targeting similar

pathways would have similar interaction profiles, we compared the similarities among drug

interaction profiles from the 17 antibiotics that target four general processes using

hierarchical clustering (Fig. 4C). Cefoxitin is removed from this analysis because there is

only one reportable combination containing cefoxitin in our dataset. In general, it is unclear

whether drug interaction profiles are clustered by the mechanism of action. For example,

rifabutin and nitrofurantoin have similar interaction profiles but different mechanisms of

action. However, there are also examples of drugs with a similar mechanism of action that

have similar interaction profiles, such as cell-wall acting agents ethambutol and cerulenin, the

fluoroquinolones levofloxacin and moxifloxacin, protein synthesis inhibitors azithromycin

and telithromycin (Fig. 4C). Nevertheless, it is possible that an analysis of drug interaction

profiles across a larger set of drugs may reveal patterns that are not present in this dataset due

to the small number of representatives for each drug class. Further analysis with a larger drug

set with an even greater number of representatives for each drug category may be necessary

to understand whether drug interaction patterns are similar in *M. abscessus* for drugs with

closely related mechanisms of action. However, our data do not suggest that there are strong

similarities in drug interactions for drugs that target the same pathways.

Drug interaction is strain-specific

An extensive study of 85 clinical isolates from *M. abscessus* subspecies demonstrated

species-specific drug susceptibility, including clinically favored drugs, such as amikacin and

macrolide clarithromycin (67). For this reason, we speculated that drug interactions would

also vary from isolate to isolate. To test this hypothesis, we selected three strains that were

chosen to represent major types of variation among strains, including colony morphology

(e.g., smooth versus rough phenotype) and growth rate (e.g., slow- versus fast-growing). The

fastest and the slowest growing strain were the lab reference strain ATCC19977 and TMC2

(4hr and 20hr doubling, respectively, Table S2). ATCC19977 and TMC3 shared similar

smooth morphologies, and TMC1 and TMC2 shared similar rough morphologies. Detailed

information about the doubling time and phenotype of these clinical isolates is included in

Table S2.

We used a focused drug set for isolate-to-isolate comparison; the set includes antibiotics that

are recommended for current therapies for NTMs and TB (amikacin, azithromycin, cefoxitin,

clarithromycin, moxifloxacin, and rifampicin), in development (SPR719 and bedaquiline), or

are broadly synergistic in ATCC19977 (telithromycin). Consistent with previous studies, we

observed variation in IC_{50} values from isolate-to-isolate (Fig. 5A) (11, 67). Though

susceptibility among strains is also variable for newer drugs, some relative potencies for

single-antibiotics are retained across strains. For example, bedaquiline and SPR719 have the

highest potency (e.g., lowest IC_{50}) for the lab reference strain, and this observation holds for

other strains (except for SPR719 in TMC3 strains, Fig. 5A). To understand if colony

morphotype or growth rate was correlated with drug susceptibility, we calculated Pearson

correlations of IC_{50} values between each strain (Fig. S4A). In general, we observed poor

correlations in single-drug susceptibility patterns between strains, even in strains with
common morphologies and similar growth rates (ATCC1977, TMC3) and (Table 2, Fig.
S4A). The only significant correlation (R = 0.88) observed is between TMC2 and
ATCC19977, which differ in both morphology and growth rate (Table 2). Together, our data
suggest that isolate-to-isolate differences in drug response are not well correlated with colony
morphology and doubling times.

Table 2. Characterization of lab strain (ATCC19977) and clinical isolates TMC1, TMC2
and TMC3

strain	doubling time (hour)	morphology
ATCC19977	4	smooth
TMC 1	12	rough
TMC 2	20	rough
TMC 3	8	smooth

A

B

$\log_2 FIC_{50}$

synergistic   additive   antagonistic

FIG 5. Single drug susceptibility and drug interaction outcomes and in different
***M. abscessus* clinical isolates.** (A) Single drug susceptibilities for the reference strain
(ATCC19977) and three clinical isolates (TMC1, TMC2, and TMC3). Susceptibility
is measured by the IC_{50} (µg/ml). Error bars represent standard deviation as compared
to the mean, when there are more than one replicates IC values are reported. (B) Heat
map showing drug interactions for each strain: ATCC19977 (top left), TMC1 (top
right), TMC2 (bottom left) and TMC3 (bottom right). As in Fig 2, drugs are organized
by the mechanism of action, and drugs recommended for treating *M. abscessus*
infection are indicated in bold text. Drug measurement is expressed in log_2FIC_{50},
where blue highlights $log_2FIC < 0$ (synergy) and red is $log_2FIC > 0$ (antagonism).

To understand whether drug interactions were also strain-dependent, we measured pairwise
drug interactions among the nine drugs in our focused antibiotics set with DiaMOND. The
drug interaction profiles varied from isolate to isolate (Fig. 5B). Correlation analysis
demonstrates that the most similarity in drug interactions is between TMC1 and ATCC19977
(R = 0.48, Fig. S4B). We noted that there was no correlation in single-drug IC_{50} values
between TMC1 and ATCC19977, nor did these two strains share morphologies or growth
rates. The lack of similarity between these strains, except in the drug interaction profile,
supports our hypothesis that drug interaction is independent of single drug potency and
morphology. The spectrum of drug interactions is balanced in ATCC19977, TMC1, and
TMC3, whereas TMC2 strongly tends toward antagonism (Fig. 5B). Despite poor overall
correlations in drug interaction profiles from strain to strain, we observed some similarities
between these strains. For example, amikacin paired with macrolides (such as azithromycin
or clarithromycin) is consistently antagonistic. In contrast, combinations of clarithromycin
and SPR719 or bedaquiline and telithromycin were all synergistic (Fig. 5B). There are
strongly synergistic combinations such as clarithromycin with almost all tested drugs for
TMC1 (except amikacin) or telithromycin with almost all tested drugs for TMC3 (except
amikacin and azithromycin) (Fig. 5B), suggesting that there may be space for improvement in
combination therapy in a strain specific manner. Taken together, our results affirm the need
for targeted drug combination testing and suggest that measurements should be made for each
isolate for reliable drug interaction determination.

DISCUSSION

Infections caused by *M. abscessus* are notoriously difficult to treat for many reasons,
including innate and acquired drug resistance, low antibiotic efficacies, and variation among

strains. Combination antibiotic treatment has the potential to improve treatment efficacies via synergies and potentiation. Despite this potential and reports of several synergistic drug combinations, we have lacked a comprehensive drug interaction dataset to develop drug combinations for the treatment of *M. abscessus*. Here, we applied DiaMOND to systematically measure pairwise drug interactions among 18 drugs against *M. abscessus* to better understand the landscape and potential of combination therapy for effective treatment. Our results include 122 drug pairs for which we report interactions for the first time. These data revealed an unexpectedly large synergy space compared to other bacterial species. We observe many synergistic drug pairs that are currently not considered as options for treating *M. abscessus* pulmonary infection in clinical practice. One example is amoxicillin, which is not currently recommended to be used to treat *M. abscessus*. Amoxicillin is synergistic with almost all tested drugs, combined with its clinical practicality suggests its potential to be used in combination therapy. We did not find that clinically used drug pairs were necessarily synergistic *in vitro*. For example, amikacin and azithromycin is antagonistic *in vitro* while another pair of protein synthesis inhibitors (azithromycin and linezolid) is synergistic. Together, the large number of synergies identified here *in vitro* motivates future investigation of these combinations as a path forward to improve combination therapy to treat pulmonary *M. abscessus* infection.

Our findings demonstrate that some drugs that are not effective alone potentiate the activities of other antibiotics. These agents may play an important role in improving *M. abscessus* regimens given the poor efficacy of antibiotics as monotherapies for *M. abscessus*. To understand whether we could sensitize *M. abscessus* to antibiotics using potentiators, we expanded our combinations screen and adapted the DiaMOND methodology to evaluate how drugs that are considered inactive affect the efficacies of active drugs. Among the four candidate potentiators we tested, avibactam has been subjected to multiple studies due to its ability to inhibit Bla_{mab} and potentially allow more ß-lactams to be used to treat *M. abscessus*. Our results are consistent with previously reported studies on combining a non-ß-lactam ß-lactamase inhibitor with ß-lactam to extend the efficacy of ß-lactam, which suggests that avibactam should be considered in a multi-drug therapy (61, 63). Additionally, the most potentiating effect is observed at 90% growth inhibition (not 50% growth inhibition), suggesting that the potentiators' effect is likely to be more prominent at higher concentrations of the active antibiotic.

We found that we could not predict drug interactions based on drug potencies or mechanisms

of action. Though drug interaction profiles were not very similar among antimicrobials

targeting the same cellular process (cell wall, DNA, etc.), we observed that some antibiotics

were consistently synergistic or antagonistic with drugs from the same class. For example,

DNA-targeting drugs are antagonistic with linezolid and respiratory inhibitors (bedaquiline

and thioridazine) and synergistic with cell-wall-acting antibiotics (ethambutol, vancomycin,

and amoxicillin). The reason for the observed synergy between cell-wall-acting and DNA-

acting compounds may be due to cell wall targeting compounds increasing the permeability

of the mycobacterial cell wall, resulting in an increased accumulation of DNA-targeting

compounds in the cell (68). *M. abscessus* is known to increase the activity of efflux pump

inhibitors when exposed to bedaquiline, which may result in the rapid efflux of DNA-acting

compounds and an antagonist interaction (23).

Another challenge with developing an effective regimen for *M. abscessus* is the remarkable

variation in drug response among strains, likely due to genomic diversity in NTM species,

even across morphologically related isolates (26, 69). We measured drug interactions in three

clinical isolates with different colony morphologies and growth rates. Previous studies have

shown that the impact of colony morphotype, which includes the transition of the smooth

colony to the rough colony during infection, highly affects drug susceptibility (70). To

understand whether a universal multidrug regimen may be developed that is effective against

all *M. abscessus* strains, we measured a core set of drug pairs in different clinical strains of *M.*

abscessus. We found that drug interactions were poorly correlated across strains and could

not be systematically determined based on data from the reference strain, strains with similar

morphologies or growth rates, the single-drug susceptibility profiles, or the drug mechanisms

of action. This result suggests the potential need to include susceptibility testing for drug

combinations, instead of single drugs only in clinical laboratory testing. Strain-to-strain

variation in drug interaction profile may be determined by subspecies or genetic differences,

for instance the presence of the *erm* gene, which can confer macrolide resistance. Future

work measuring drug interactions across a large set of clinical isolates is required to address

this question. Nevertheless, our observations drug interactions in *M. abscessus* are strain-

specific supports the idea that treatment regimen development may need to be personalized

for each isolate (e.g., directly measured) rather than derived from guidelines based on other

strains.

This systematic study of the drug combination landscape in *M. abscessus* suggests that we

require a more extensive phenotypic evaluation of drug interactions to develop improved

therapies. Additionally, the relationship between *in vitro* single drug susceptibility or

combination measurement and clinical outcome in *M. abscessus* has not yet been sufficiently

studied, and it is currently unclear whether synergy correlates with positive clinical outcomes

(71). This challenge in linking *in vitro* measurement to *in vivo* response is not unique to

NTMs and is well characterized in tuberculosis (72, 73). However, previous studies have

shown that measurement of the drug combination response across multiple growth conditions

that resemble the host's microenvironment is predictive of treatment shortening in Mtb (30,

74). Environmental nutrients have a critical effect on drug interaction in both *M. abscessus*

and Mtb, and antibiotic response is altered when grown in artificial cystic fibrosis sputum (30,

75, 76). We, therefore, expect that making drug interaction measurements in different host-

mimicking growth conditions and testing which of these growth environments predicts

treatment outcomes in preclinical animal models and in humans is a critical step in drug

combination design for *M. abscessus* and other NTMs. Together, the dependence of drug

interactions on strain and variation in drug response with strain and growth conditions

suggests that we have failed to identify a universal combination therapy that is effective for

M. abscessus not because good drug combinations do not exist, but rather because we need to

better understand how to tailor combination therapy to each infection.

MATERIALS AND METHODS

Antimicrobials

The antimicrobials agents used in this study (except for SPR719) were obtained from Sigma

Aldrich. SPR719 was a gift from Spero Therapeutics. Stock solutions were prepared in

DMSO or sterile water + 0.01% Triton X-100, depending on solubility, and stored in single-

use aliquots at -20°C until used.

Strain and culturing

Measurement was made using *M. abscessus* subsp. *abscessus* strain ATCC19977 (reference

strain) and clinical isolates obtained from patient sputum at Tufts Medical Center, Infectious

Disease Clinic (TMC1, TMC2, and TMC3) (77). All strains were cultured in a 7H9 medium

supplemented with 0.05% Tween 80, 0.2% Glycerol, and 10% BBL Middlebrook ADC

enrichment. Cultures were started from frozen aliquots and allowed to grow to the mid-log

phase (OD_{600} between 0.4 - 0.6), shaking at 37°C overnight. Cultures were then diluted once

to the lag phase (OD_{600} between 0.05 - 0.1) and allowed to grow to the mid-log phase before

performing assays.

740   DiaMOND measurement

DiaMOND was used to measure drug interactions, as previously described for

Mycobacterium tuberculosis (18). Details of DiaMOND can be obtained in Mycobacteria

Protocol 4th, chapter 30th (78). Briefly, DiaMOND uses equipotent drug-combination dose-

response curves to approximate the shape of checkerboard isoboles at the same level of

growth inhibition. Minor adjustments (e.g., the dose-response curve is centered around 50%

inhibitory concentration (IC_{50}) in contrast to 90% inhibitory concentration (IC_{90}) for *M.*

tuberculosis, the modification of fractional inhibition concentration to fold shifts in inhibition

concentration) were made to account for the rapid growth of *M. abscessus* and low drug

potency relative to *M. tuberculosis*. Details of these adjustments are explained in subsequent

sections.

Growth inhibition assay

Antibiotics tested were either dissolved in dimethyl sulfoxide (DMSO) or sterile water + 0.01%

Triton X-100 and stored at -20˚C in single-use aliquots. Assays were performed in clear, flat

bottom 384-well microplates. Drugs were dispensed using a digital drug dispenser (D300e

Digital Dispenser, HP). Drug wells were randomized across plates to minimize plate position

effects. Bacterial cultures were diluted to OD_{600} of 0.05 in fresh medium, and 50µL of diluted

culture was added to each well for drug treatment. Plates were sealed with optically clear

plate seals and incubated without shaking at 37˚C. Growth (OD_{600}) was measured by a

microplate reader (BioTek) 48h after drug treatment.

Data analysis

OD_{600} data were processed using MATLAB's custom analysis pipeline (MathWorks). Data

were first derandomized from the plate layout, and dose-response curves were organized for

each drug and drug combination. The first row of wells in each 384-well plate contained

medium-only wells. The median of these medium-only wells was subtracted from each well

in a plate as a background. Drug-treated wells were normalized to the mean of the untreated

wells (controls), and the values obtained were subtracted from 1 to get a dose-response

inhibition curve where 0 and 1 represented no growth inhibition and full growth inhibition,

respectively. Growth inhibition curves were then fit to a three-parameter Hill curve using a

nonlinear solver in MATLAB (79). The fit accuracy was assessed using the R^2 metric.

Equations derived from the Hill function were used to calculate different inhibitory

concentration values (IC values) along the dose-response curve.

Drug interaction calculation

To assess whether drugs in combination were synergistic, additive, or antagonistic, we

measured the fractional inhibitory concentrations (FIC). We used Loewe additivity as the null

model to calculate the FIC values (80). We calculated the expected IC value of the

combination AB (drug A with drug B) from the intersection of the combination line to the

line of additivity determined by the IC values of A and B alone (Fig. 1A). Finally, the FIC

value is calculated by dividing the experimentally observed IC by the expected IC value of

the drug combination.

780
$$FIC = \frac{observed\ IC}{expected\ IC}$$

We report the $\log_2$ of the FIC so that the magnitude of synergy and antagonism scores are

balanced around zero. A $\log_2$FIC value < 0 is synergistic, $\log_2$FIC value $\sim= 0$ is additive, and

$\log_2$FIC value > 0 is antagonistic. FIC scores were calculated at IC_{50} (FIC_{50}) and IC_{90} (FIC_{90}).

With DiaMOND, we can measure drug interaction scores using other null models such as

Bliss independence. By Bliss independence, the expected IC value for [AB] is calculated by

multiplying drug A's effect and drug B's effect at the desired IC level.

Certain drugs were tested at a fixed concentration (instead of increasing doses) due to their

lack of inhibitory effect as a single agent. These drugs are also referred to as potentiator

candidates. The concentration of the potentiator candidates was estimated from previously

reported serum concentrations. To quantify drug interaction scores for potentiators, fold shifts

in ICs (FsICs) were calculated as a ratio of the observed IC_{50} value of a drug pair

combination to the IC_{50} of the active, non-potentiating drug (Fig. 1B). $\log_2$FsIC < 0 are

potentiated whereas $\log_2$FsIC > 0 are attenuated.

Quality control

Assay quality was assessed in three different ways. The Z-factor was used to determine the

quality of the assay at the plate level. For a given plate, the Z-factor was calculated as:

$$Z' = 1 - \frac{3(\sigma_{positive} + \sigma_{negative})}{|\mu_{positive} - \mu_{negative}|}$$

where σ is the standard deviation of the positive and negative control (untreated cells), μ is

the mean of the positive and negative controls. Plates with a Z' between 0.5 - 1 indicate

excellent assay with a statistically reliable separation between positive and negative control.

The second level of assessment was at the dose-response level. The R^2 value derived from fits

of the dose-response curves to Hill curves was used to determine fit accuracy. We combined

R^2 values with a visual inspection of the fits. Any Hill curve fits with R^2 values below 0.7

were marked as poor fits and rejected from further analysis. Because of the intrinsic

resistance property of *M. abscessus*, it is challenging to obtain consistent dose-response

curves (81). For example, to capture data points close enough to each other to draw an

accurate dose response, we need to design doses to increase by 1.5x instead of 2c, which

limits the testing range. In addition, the noise of the dose-response curve made fitting

challenging. Finally, obtaining a maximum inhibitory concentration (MIC) for all drugs is

difficult due to the heterogeneity of *M. abscessus* (81). To overcome these difficulties, we

designed the experiment around IC_{50} (instead of IC_{90}), which is achieved for most drugs and

is more reproducible than IC_{90} (Table S1). To assess whether doses were sampled in an

equipotent manner for combination dose-response, the angle of the combination dose-

response was calculated (the ideal angle is 45˚). Angle deviation beyond 22.5˚ of this ideal

equipotent dose-response was deemed too far for the approximation of isoboles in the

checkerboard and was eliminated from the analysis.

Clinical isolate growth rate measurement

To measure the growth rate of clinical isolates obtained from patient sputum at the Tufts

Medical Center Infectious Disease Clinic (TMC1, TMC2, and TMC3), suspended culture of

these isolates were grown in a 7H9 medium supplemented with 0.05% Tween 80, 0.2%

Glycerol, and 10% BBL Middlebrook ADC enrichment. Cultures were started from frozen

aliquots and allowed to grow to the mid-log phase (OD_{600} between 0.4 - 0.6), shaking at 37˚C

overnight. Cultures were then diluted once to the lag phase and allowed to grow to the mid-

log phase again before performing the assay. Growth rate measurement was performed in a

96-well microplate, with five biological replicates per isolate. Reference strain ATCC19977

was also included for reference purposes. 150µL of culture at OD_{600} around 0.05 was

dispensed into each well. The microplate was sealed with an optically clear plate seal and

incubated inside a plate reader at 37˚C. OD_{600} was recorded for 18 hours in 30 minutes

intervals. Doubling time was calculated using OD_{600} values closest to the log phase. Doubling

time was calculated as:

$$r = \frac{\ln(OD'_{600} - OD_{600})}{(T' - T)}$$

$$Doubling\ time = \frac{ln2}{r}$$

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AUTHOR CONTRIBUTION

NV, YD, JLF, TS, and BBA conceived and designed the experiments. NV, YD, JK, and PL performed the experiments. NV, YD, and BBA conceived and designed the computational analysis. NV and YD performed the computational analysis. The manuscript was written by NV, YD, TS, and BBA. All authors contributed to the technical interpretation, interpretation of the results, and the editing of the manuscript.

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