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Select β-Lactam Combinations Exhibit Synergy against *Mycobacterium abscessus In Vitro*

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ABSTRACT *Mycobacterium abscessus* is a nontuberculous mycobacterium that causes invasive pulmonary infections in patients with structural lung disease. *M. abscessus* is intrinsically resistant to several classes of antibiotics, and an increasing number of strains isolated from patients exhibit resistance to most antibiotics considered for treatment of infections by this mycobacterium. Therefore, there is an unmet need for new regimens with improved efficacy to treat this disease. Synthesis of the essential cell wall peptidoglycan in *M. abscessus* is achieved via two enzyme classes, L,D- and D,D-transpeptidases, with each class preferentially inhibited by different subclasses of β-lactam antibiotics. We hypothesized that a combination of two β-lactams that comprehensively inhibit the two enzyme classes will exhibit synergy in killing *M. abscessus*. Paired combinations of antibiotics tested for *in vitro* synergy against *M. abscessus* included dual β-lactams, a β-lactam and a β-lactamase inhibitor, and a β-lactam and a rifamycin. Of the initial 206 combinations screened, 24 pairs exhibited synergy. A total of 13/24 pairs were combinations of two β-lactams, and 12/24 pairs brought the MICs of both drugs to within the therapeutic range. Additionally, synergistic drug pairs significantly reduced the frequency of selection of spontaneous resistant mutants. These novel combinations of currently available antibiotics may offer viable immediate treatment options against highly-resistant *M. abscessus* infections.

KEYWORDS β-lactamase inhibitor, β-lactams, *Mycobacterium abscessus*, antibiotics, avibactam, rifamycins, synergy

Mycobacterium abscessus is considered to be among the most virulent of the rapidly growing nontuberculous mycobacteria (NTM). It may be environmentally or nosocomially acquired (1) and can lead to severe and invasive pulmonary infections in immunocompromised patients or those with structural lung diseases, such as bronchiectasis or cystic fibrosis (CF). In the CF population, invasive *M. abscessus* infections are associated with rapid lung function decline (2–4), so adequate treatment of these infections is paramount. *M. abscessus* is intrinsically resistant to several classes of antibiotics, and the percentage of clinical isolates exhibiting resistance to the few drugs currently available to treat this infection is steadily increasing (5–8). Sputum culture conversion rates as low as 25% have been described with antibiotic treatment alone (9), and the cure rate for *M. abscessus* pulmonary disease is only 30 to 50% (10).

The current treatment guidelines for *M. abscessus* pulmonary disease include at least 18 months of multidrug therapy, several of which require intravenous administration and may be associated with significant cytotoxicity (11, 12). These recommendations are largely based on empirical evidence, as few systematic clinical trials have been performed to elucidate the optimal therapeutic regimen against *M. abscessus*. It is also frequently necessary in clinical practice to tailor treatment regimens based on the resistance profiles of individual *M. abscessus* isolates, as the high degree of variability in

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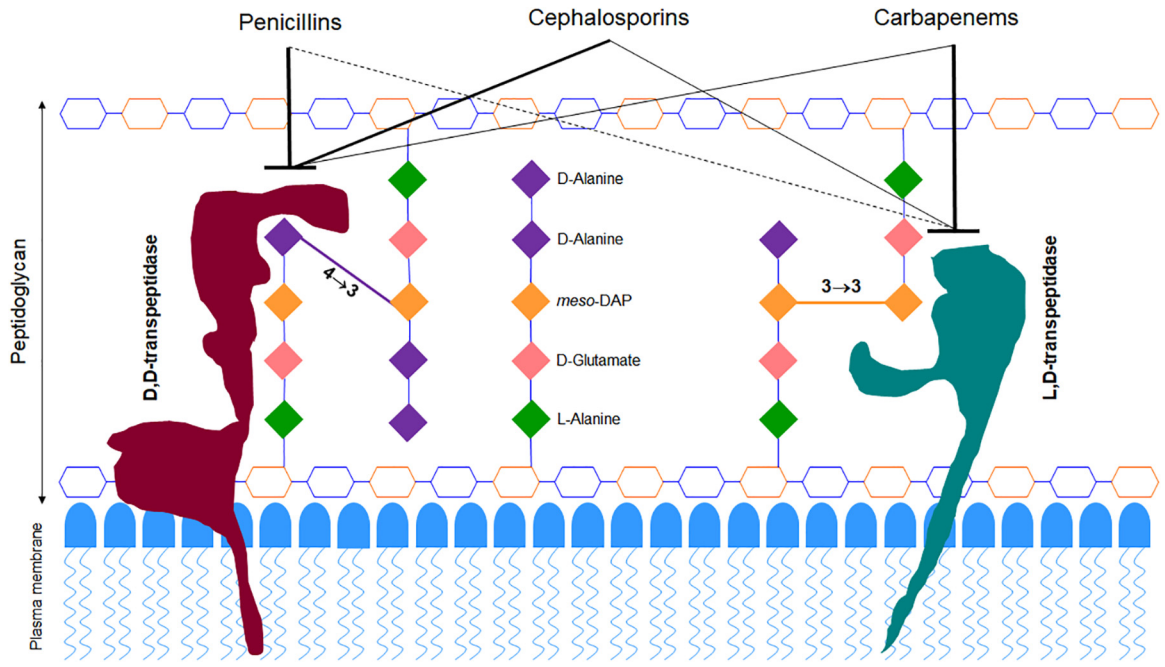

FIG 1 Model of *M. abscessus* peptidoglycan depicting preferential binding of β-lactam subclasses. The hexagonal structures represent sugars *N*-acetylglucosamine (blue) and *N*-acetylmuramic acid (orange).

antibiotic resistance observed among *M. abscessus* strains often precludes the use of a standardized regimen (10).

Macrolide antibiotics have historically been considered the backbone of treatment against many NTM, including *M. abscessus* (2, 12). However, two of the three *M. abscessus* subspecies, *Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *bolletii*, harbor a functional *erm*(41) gene, which confers inducible macrolide resistance and, thus, limits the effectiveness of this antibiotic class beyond the first 2 weeks of treatment (13, 14). Guidelines, therefore, recommend subspeciation of the *M. abscessus* complex, which many clinical laboratories are not equipped to perform routinely. Consequently, some CF centers prescribe initial treatment regimens that include a combination of intravenous amikacin and either cefoxitin or imipenem rather than a macrolide (15). Cefoxitin (a cephalosporin) and imipenem (a carbapenem) are the only two β-lactam antibiotics included in the current *M. abscessus* treatment guidelines (12, 16).

β-lactams function by inhibiting enzymes that catalyze synthesis of peptidoglycan, a three-dimensional macromolecule that forms the exoskeleton of bacterial cells (17). During the final step of peptidoglycan synthesis, *M. abscessus* utilizes two enzyme classes, the canonical D,D-transpeptidases (DDTs) (also known as penicillin-binding proteins) and the recently discovered L,D-transpeptidases (LDTs) (18), to generate 4→3 and 3→3 linkages between stem peptides, respectively (Fig. 1). Since as many as 80% of the linkages in *M. abscessus* peptidoglycan are of the 3→3 type (18), the LDTs that generate them are likely at least as important as DDTs for this organism. An initial survey of the *M. abscessus* genome identified five putative LDT-encoding genes (19). These LDTs are differentially susceptible to β-lactam subclasses, with most carbapenems exhibiting strong inhibitory activities, followed by cephalosporins and only a few penicillins exhibiting moderate inhibition of these enzymes (20, 21).

Inhibition of peptidoglycan synthesis is lethal to bacteria (22). As LDT and DDT activities are required for synthesis of *M. abscessus* peptidoglycan, simultaneous inhibition of both enzymes could be bactericidal. Since these enzymes exhibit differential susceptibilities to β-lactams (21, 23, 24), we hypothesize that a combination of β-lactam subclasses—one that optimally inhibits LDTs and another that specifically targets

TABLE 1 MICs of individual antibiotics tested against *M. abscessus* ATCC 19977 *in vitro*

β-Lactam class	Drug	MIC (μg/ml)
Cephalosporins	Cefadroxil	256
	Cefprozil	256
	Cefuroxime	256
	Cefixime	256
	Ceftibuten	256
	Cefdinir	64
	Cefditoren	256
	Cefpodoxime	256
	Cefoxitin	64
Carbapenems	Ertapenem	256
	Meropenem	32
	Imipenem	8
	Doripenem	16
	Biapenem	16
	Tebipenem	256
Penem	Faropenem	256
Rifamycins	Rifabutin	32
	Rifapentine	128
	Rifampin	128
β-Lactamase inhibitors	Avibactam	256
	Clavulanate	256

DDTs—will demonstrate synergy in killing *M. abscessus*. In this study, we have tested this hypothesis by assessing the potencies of combinations of 16 β-lactams consisting of cephalosporins and carbapenems against *M. abscessus*. Penicillins were not assessed, as many require frequent dosing, and we preferentially chose oral cephalosporins requiring once or twice daily dosing to simplify administration in patients.

M. abscessus exhibits robust β-lactamase activity via Bla_{Mab}, which significantly reduces the efficacy of β-lactams against this mycobacterium (25, 26). Bla_{Mab} degrades several β-lactams with much greater efficiency than BlaC in *Mycobacterium tuberculosis* (27). Among the known β-lactamase inhibitors, avibactam strongly inhibits Bla_{Mab} (28) and reduces the MICs of various β-lactams against *M. abscessus* (25, 29–31). Although clavulanate, tazobactam, and sulbactam are not potent inhibitors of Bla_{Mab} (27), clavulanate is the only orally bioavailable agent, and whether it exhibits synergy in combination with β-lactams against *M. abscessus* is not sufficiently documented. Therefore, we have included avibactam and clavulanate in our study. Rifamycins were also included based on prior demonstration of synergy between carbapenems and rifamycins against *M. abscessus in vitro* (32–34).

RESULTS

MICs of β-lactams against *M. abscessus*. We evaluated the antimicrobial activity of several β-lactam antibiotics, including nine cephalosporins (cefadroxil, cefprozil, cefuroxime, cefixime, ceftibuten, cefdinir, cefditoren, cefpodoxime, and cefoxitin), six carbapenems (ertapenem, meropenem, imipenem, doripenem, biapenem, and tebipenem), and a penem (faropenem), by determining their MICs against *M. abscessus* (Table 1). We preferentially tested oral cephalosporins that did not require more than twice daily dosing, as their use in the clinical setting would be more convenient. MICs were also determined for three rifamycin antibiotics (rifabutin, rifapentine, and rifampin) and two β-lactamase inhibitors (clavulanate and avibactam), which were tested for synergy with β-lactams in subsequent experiments. The majority of cephalosporins exhibited high baseline MICs of 256 μg/ml, with the exception of cefoxitin and cefdinir at 64 μg/ml. The MICs of the carbapenems and penem were more variable, ranging from 8 μg/ml for imipenem to 256 μg/ml for ertapenem, tebipenem, and faropenem.

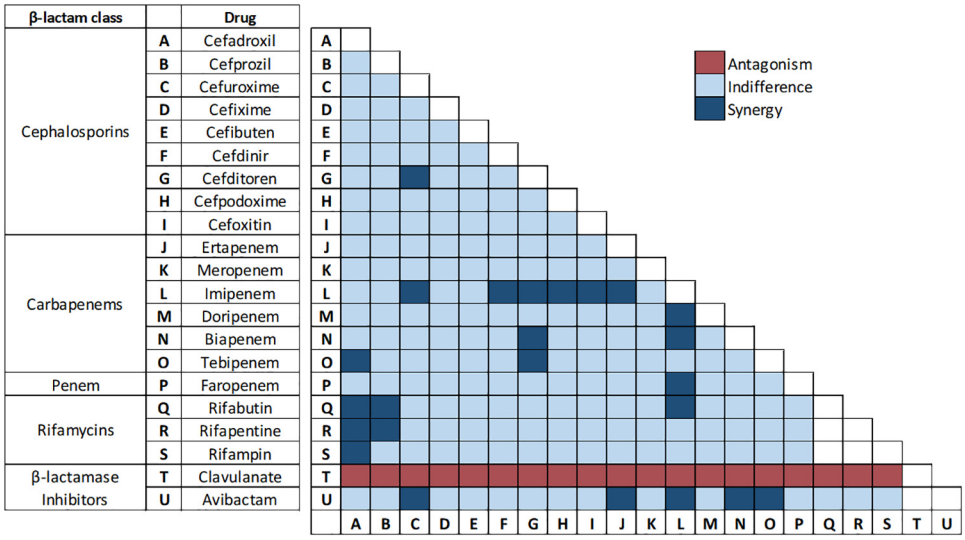

FIG 2 Results representing potencies of dual drug combinations against *M. abscessus* using checkerboard assay. Each box represents a combination of drugs shown in the *x* axis and *y* axis. Inhibition of *M. abscessus* growth in samples containing drug pairs at one-fourth MIC or less of each drug is designated "Synergy" and growth at one-half to 1× MIC of each drug is designated "Indifference." Growth of *M. abscessus* in samples containing drug pairs at 2× MIC of each drug is designated "Antagonism." These designations are based on the published guidelines for interpreting checkerboard assay results (35–37). Combinations that were not assessed are represented by blank boxes.

Several β-lactam combinations exhibit synergy against *M. abscessus*. A total of 206 paired combinations of antibiotics were initially screened for synergy via a condensed version of the checkerboard assay as described in Materials and Methods. Combinations included all possible pairs of cephalosporins and carbapenems/penem, a rifamycin with either a cephalosporin or carbapenem/penem, and avibactam or clavulanate with a cephalosporin or carbapenem/penem (Fig. 2).

Of the initial 206 combinations screened, 24 combinations showed no growth at one-fourth of the MIC or less for each drug and were further evaluated to verify synergy and determine the fractional inhibitory concentration index (FICI) using a checkerboard titration assay as described in Materials and Methods (Fig. 2). The FICI of a synergistic pair is a mathematical representation of the degree to which each drug contributes to synergy (35–37). These 24 synergistic combinations included cefuroxime and avibactam, biapenem and avibactam, cefoxitin and imipenem, cefditoren and imipenem, imipenem and doripenem, cefuroxime and cefditoren, cefditoren and biapenem, cefdinir and imipenem, cefuroxime and imipenem, cefpodoxime and imipenem, imipenem and biapenem, imipenem and avibactam, tebipenem and avibactam, ertapenem and avibactam, ertapenem and imipenem, imipenem and faropenem, imipenem and rifabutin, cefadroxil and tebipenem, cefditoren and tebipenem, cefadroxil and rifabutin, cefadroxil and rifapentine, cefadroxil and rifampin, cefprozil and rifabutin, and cefprozil and rifapentine (Table 2). Although substantial variability in the level of synergy among paired combinations was observed, there were a few notable trends. For example, imipenem was synergistic with the majority of drugs it was tested with, and the rifamycins were synergistic with select earlier generation cephalosporins. Of note, all of the combinations that included clavulanate failed to inhibit growth of *M. abscessus* in the presence of antibiotics at concentrations as high as 2× MIC. This antagonism was unexpected but was reliably reproducible when the experiment was repeated.

To determine if the degree of synergy between paired combinations was sufficient to reduce MICs to within the therapeutic range, the fractional inhibitory concentration (FIC) of each drug in combination was used to extrapolate expected MICs as a result of synergy. Although Clinical and Laboratory Standards Institute (CLSI) guidelines regarding MIC breakpoints against *M. abscessus* are not currently available for most of the

TABLE 2 Fractional inhibitory concentration indices of synergistic drug pairs[a]

Drug combination	MIC of single drug (μg/ml)	MIC in combination (μg/ml)	FICI
Cefuroxime and avibactam	256/256	32/5	0.15
Biapenem and avibactam	16/256	4/4	0.27
Cefoxitin and imipenem	64/8	9/1	0.27
Cefditoren and imipenem	256/8	26/1	0.27
Imipenem and doripenem	8/16	2/2	0.30
Cefuroxime and cefditoren	256/256	33/44	0.30
Cefditoren and biapenem	256/16	26/4	0.32
Cefdinir and imipenem	64/8	9/2	0.35
Cefuroxime and imipenem	256/8	30/2	0.35
Cefpodoxime and imipenem	256/8	28/2	0.36
Imipenem and biapenem	8/16	2/3	0.42
Imipenem and avibactam	8/256	2/64	0.47
Tebipenem and avibactam	256/256	28/5	0.13
Ertapenem and avibactam	256/256	64/4	0.27
Ertapenem and imipenem	256/8	19/2	0.29
Imipenem and faropenem	8/256	1/29	0.29
Imipenem and rifabutin	8/32	2/3	0.31
Cefadroxil and tebipenem	256/256	40/48	0.34
Cefditoren and tebipenem	256/256	64/32	0.38
Cefadroxil and rifabutin	256/32	48/6	0.38
Cefadroxil and rifapentine	256/128	64/16	0.38
Cefadroxil and rifampin	256/128	64/32	0.50
Cefprozil and rifabutin	256/32	64/8	0.50
Cefprozil and rifapentine	256/128	64/32	0.50

[a]Combinations capable of reducing MICs to within susceptible/intermediate range based on established/presumed breakpoints are listed on the top half of the table in order of ascending FICI. FICIs and MICs in combination were extrapolated using data averaged from 2 to 3 replicate experiments.

antibiotics tested, they have been established for cefoxitin and imipenem (38). Therefore, MIC breakpoints for all cephalosporins and carbapenems/penem were assumed to be the same as those for cefoxitin (≤16 μg/ml, susceptible; ≤64 μg/ml, intermediately susceptible) and imipenem (≤4 μg/ml, susceptible; ≤8 μg/ml, intermediately susceptible), respectively. With regard to the rifamycins, a breakpoint MIC of ≤1 μg/ml has been established for rifampin and rifabutin against *M. tuberculosis* and *Mycobacterium avium* complex (38), and the same principle was applied. The most recent guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) do not include clinical breakpoints of antibiotics against *M. abscessus* (39). Based on these presumed breakpoints, 5/24 combinations exhibited MICs within the fully susceptible range for both drugs. They are biapenem and avibactam, cefoxitin and imipenem, imipenem and doripenem, cefdinir and imipenem, and imipenem and biapenem. An additional 7/24 combinations showed MICs that were considered moderately susceptible or intermediate. They are cefuroxime and avibactam, cefditoren and imipenem, cefuroxime and cefditoren, cefditoren and biapenem, cefuroxime and imipenem, cefpodoxime and imipenem, and imipenem and avibactam. The remaining 12/24 combinations were unable to bring MICs below resistance breakpoints.

All of the 12 most synergistic combinations—those that brought MICs within the therapeutic range—were pairs of either two β-lactams or a β-lactam and avibactam. Several of the remaining 12/24 combinations also exhibited a high degree of synergy based on FICI; however, their initial MICs were so high that the synergistic effect was insufficient to reduce MICs to below presumed breakpoints. We also observed a limit to the degree of synergy achievable with drugs exhibiting relatively low initial MICs, such as imipenem, biapenem, and doripenem. Combinations with these drugs resulted in up to a 4-fold decrease in MIC, but this appeared to be the limit of reduction as MICs approached 2 μg/ml.

Additionally, the rifamycins showed synergy in combination with two of the earlier generation cephalosporins (cefprozil and cefadroxil), with at least a 4-fold reduction in MIC for each combination. However, given the relatively low presumed MIC breakpoint

TABLE 3 Frequency of emergence of spontaneous drug-resistant mutants of *M. abscessus* when exposed to individual drugs and paired combinations that exhibit synergy *in vitro*

Drug combination	Resistance frequency for:	
	Individual drug	Combination
Cefuroxime and avibactam	$>2 \times 10^{-6}$/—	$<1 \times 10^{-10}$
Biapenem and avibactam	8.9×10^{-8}/—	3.3×10^{-9}
Cefoxitin and imipenem	$>2 \times 10^{-6}/1.9 \times 10^{-7}$	$<1 \times 10^{-10}$
Cefditoren and imipenem	$>2 \times 10^{-6}/1.9 \times 10^{-7}$	$<1 \times 10^{-10}$
Imipenem and doripenem	$1.9 \times 10^{-7}/9.9 \times 10^{-8}$	9.1×10^{-9}
Cefuroxime and cefditoren	$>2 \times 10^{-6}/>2 \times 10^{-6}$	$<1 \times 10^{-10}$
Cefditoren and biapenem	$>2 \times 10^{-6}/8.9 \times 10^{-8}$	$<1 \times 10^{-10}$
Cefdinir and imipenem	$7.8 \times 10^{-9}/1.9\times 10^{-7}$	$<1 \times 10^{-10}$
Cefuroxime and imipenem	$>2 \times 10^{-6}/1.9 \times 10^{-7}$	$<1 \times 10^{-10}$
Cefpodoxime and imipenem	$>2 \times 10^{-6}/1.9 \times 10^{-7}$	$<1 \times 10^{-10}$
Imipenem and biapenem	$1.9 \times 10^{-7}/8.9 \times 10^{-8}$	1.1×10^{-9}
Imipenem and avibactam	1.9×10^{-7}/—	$<1 \times 10^{-10}$
Tebipenem and avibactam	1.1×10^{-7}/—	$<1 \times 10^{-10}$
Ertapenem and avibactam	8.3×10^{-8}/—	$<1 \times 10^{-10}$
Ertapenem and imipenem	$8.3 \times 10^{-8}/1.9 \times 10^{-7}$	4.3×10^{-9}
Imipenem and faropenem	$1.9 \times 10^{-7}/<1 \times 10^{-10}$	$<1 \times 10^{-10}$
Imipenem and rifabutin	$1.9 \times 10^{-7}/<1 \times 10^{-10}$	$<1 \times 10^{-10}$
Cefadroxil and tebipenem	$>2 \times 10^{-6}/1.1 \times 10^{-7}$	1.3×10^{-8}
Cefditoren and tebipenem	$>2 \times 10^{-6}/1.1 \times 10^{-7}$	$<1 \times 10^{-10}$
Cefadroxil and rifabutin	$>2 \times 10^{-6}/<1 \times 10^{-10}$	$<1 \times 10^{-10}$
Cefadroxil and rifapentine	$>2 \times 10^{-6}/<1\times 10^{-10}$	$<1 \times 10^{-10}$
Cefadroxil and rifampin	$>2 \times 10^{-6}/>2 \times 10^{-6}$	$<1 \times 10^{-10}$
Cefprozil and rifabutin	$>2 \times 10^{-6}/<1 \times 10^{-10}$	$<1 \times 10^{-10}$
Cefprozil and rifapentine	$>2 \times 10^{-6}/<1 \times 10^{-10}$	$<1 \times 10^{-10}$

of $\leq$1 μg/ml for rifamycins, none of the combinations brought MICs within the therapeutic range.

***β*-lactam combinations reduce frequency of selection of spontaneous drug-resistant mutants.** The 24 synergistic combinations were also evaluated to determine the frequency at which spontaneous resistant mutants are selected in the presence of paired drug combinations compared to that for each drug alone (Table 3). The frequency of resistant mutant selection was lower for all 24 combinations than the frequency for each drug individually. The greatest decrease was noted among the cephalosporins at >4 log reduction for six out of seven agents, with cefdinir being the exception, as the mutation frequency of this drug alone was lower than that of the other cephalosporins. Also of note, rifapentine and rifabutin exhibited the lowest frequency of resistant mutant selection, with no mutant colonies observed on any of the individual drug plates.

DISCUSSION

Given the growing prevalence of extensively drug-resistant *M. abscessus* infections, development of novel treatment strategies is imperative. Although our current understanding of β-lactam targets in *M. abscessus* is not comprehensive, we have leveraged the available data to begin developing synergistic treatment regimens with potential to treat *M. abscessus* infections that are resistant to standard therapies.

In this study, we tested a total of 206 paired combinations of antibiotics *in vitro* against *M. abscessus* reference strain ATCC 19977, 24 of which displayed synergy with FICIs of $\leq$0.5. Of these, 12 combinations achieved MIC reductions below presumed breakpoints for both drugs. Although a few studies have been published describing synergy between β-lactams and other antibiotic classes against *M. abscessus in vitro* (32, 34, 40, 41), only one has assessed synergy between dual β-lactams (21). Our current study encompasses a broader array of combinations and offers a more comprehensive analysis of the synergistic activity of the two major β-lactam subclasses currently used to treat *M. abscessus* infections: cephalosporins and carbapenems.

We hypothesize that the basis for synergy exhibited by β-lactam pairs is their nonredundant selective inhibition of distinct transpeptidases that synthesize pepti-

doglycan in *M. abscessus*. If only one type of enzyme existed as the target for β-lactams, the pairs would likely exhibit additive activity rather than synergy. Variation in the level of inhibition of LDTs of *M. abscessus* (21) and *M. tuberculosis* (20, 24, 42) by agents of this antibiotic class supports this hypothesis. We further hypothesize that, at the molecular level, the structures of β-lactams that exhibit synergy against *M. abscessus* most effectively complement the structure of the binding sites available in the transpeptidases of this organism, thereby favoring initial binding and subsequent interaction to bring about effective inhibition of the enzymes. Differences in binding affinities and kinetics of inhibition by different β-lactams against the LDTs of *Enterococcus faecium* (43) illustrate the basis for this hypothesis. By virtue of belonging to the β-lactam group, the penicillin subclass likely also interacts with the transpeptidases of *M. abscessus*, and some drugs in this class may potentially inhibit them as they are known to inhibit DDTs in other organisms. To develop a comprehensive understanding of interactions of the entire β-lactam class against their targets in *M. abscessus*, inclusion of the penicillins is necessary. The scope of the current study was limited to the β-lactam subclasses with demonstrated potential for clinical utility in treating *M. abscessus* infections. Penicillins were not included, as several members of this subclass require frequent dosing, making them less suitable for treating chronic diseases, such as *M. abscessus* infection. The number of proteins encoded by the *M. abscessus* genome with DDT activity and their identities are not known. Determination of this information would facilitate the assessment of the relationship between β-lactam subclasses and DDTs.

Synergy was also observed between the rifamycins and two of the earlier generation cephalosporins, cefadroxil and cefprozil. Although rifamycins tend to affect intracellular processes via inhibition of RNA polymerase activity, studies in *M. tuberculosis* have demonstrated synergy between rifampin and cephalosporins, especially early generation (44). It was proposed that, by damaging the cell wall, cephalosporins promote penetration by rifampin, leading to higher effective concentrations. Rifampin may also increase susceptibility to lower intracellular levels of β-lactams (44). However, rifabutin exhibited negligible synergy in combination with cephalosporins against *M. tuberculosis*, and it was hypothesized that rifabutin's lipophilicity allows for rapid penetration into the cell, thus, bypassing synergistic cell wall interactions with β-lactams. A prior study evaluating rifamycin efficacy against *M. abscessus* (33) found that rifabutin alone was active against multiple *M. abscessus* isolates, whereas rifampin showed little activity. The reasons for the differential efficacies of rifamycins against *M. abscessus* have yet to be confirmed but may be related to differences in bacterial uptake/efflux or drug metabolism (33, 45–48).

Five out of 24 synergistic combinations included avibactam. In these combinations, the MICs of three β-lactams (cefuroxime, imipenem, and biapenem) were reduced to below therapeutic breakpoints. Therefore, avibactam appears to be a viable adjunct to β-lactam-based treatment regimens. However, its current coformulation with ceftazidime (which itself does not exhibit valuable activity against *M. abscessus* [28, 30]) and intravenous administration limit its usefulness. Relevant to the strategy of combining a carbapenem with a β-lactamase inhibitor against *M. abscessus*, several coformulated agents have recently been developed. These include FDA-approved meropenem-vaborbactam (Melinta Therapeutics) and imipenem-relebactam (Merck), which recently completed phase III trials. Coformulations of cefepime-zidebactam (Wockhardt) and meropenem-nacubactam (Roche) are currently in phase II of development. Although these compounds have yet to be tested against *M. abscessus*, our data suggest that they could be viable options for treatment of *M. abscessus* infections and would potentially simplify β-lactam-based regimens. Interestingly, clavulanate was found to be antagonistic in combination with all β-lactams tested in this study, although the mechanism for this is unclear. As there is no precedent for antagonism of β-lactam and clavulanate combinations against other bacteria, we speculate the following hypotheses. Clavulanate is metabolized by *M. abscessus*, and the resulting metabolite alters the rate of influx or efflux of β-lactams, thereby reducing the effective concentration

available to bind target enzymes. It is also possible that binding of the clavulanate metabolite to target enzymes may alter their binding kinetics to β-lactams. In addition, the possibility of the clavulanate metabolite directly competing for binding sites associated with the 4-carbon core ring of β-lactams cannot be ruled out. Further study would be necessary to elucidate the underlying mechanism for this phenomenon, including possibilities not considered above.

Another measure of synergy is the frequency of selection of spontaneous resistant mutants. For a synergistic pair, the frequency of resistant mutant selection would ideally be lower than the product of frequencies associated with either drug alone. The majority of drug pairs with the lowest FICIs selected resistant mutants with a frequency of $<1 \times 10^{-10}$, which approaches the product of the individual drugs (Table 3). However, due to physical limitations of the number of *M. abscessus* CFU that could be used to identify resistant mutants, we were unable to obtain the exact frequency of mutant selection for several paired combinations. Based on these observations, we propose that the majority of pairs identified here exhibit synergy in both antimicrobial activity and reduction of selection of drug-resistant mutants.

Although significant variability in MIC exists among *M. abscessus* strains and *in vitro* drug susceptibility data do not always correlate with clinical efficacy (49), the novel β-lactam combinations identified here using the reference *M. abscessus* strain ATCC 19977 could be leveraged for further preclinical assessment, including *in vivo* efficacy against drug-resistant clinical isolates. As *M. abscessus* treatment generally necessitates a regimen consisting of at least 3 to 4 agents, the addition of other antibiotic classes or β-lactamase inhibitors to these synergistic β-lactam combinations may further potentiate MIC reduction and improve efficacy. This may also allow clinicians to avoid use of the more cytotoxic antibiotics, such as amikacin, especially in the context of prior adverse effects.

Based on current trends of *M. abscessus* strains isolated in clinics, resistance to an increasing number of drugs is likely to continue over the next several years, further compromising our ability to treat disease resulting from this pathogen. New antibiotics and coformulations are currently in development, but none are primarily intended for treatment of *M. abscessus* or other NTM infections, and it could take several years for efficacy studies to be completed. Repurposing currently available antibiotics in novel combinations, such as those identified here, may provide vital immediate therapeutic options for patients failing standard *M. abscessus* treatment regimens and facilitate rapid implementation in the clinical setting.

MATERIALS AND METHODS

Bacterial strains and *in vitro* growth conditions. The *M. abscessus* reference strain ATCC 19977 (ATCC, Manassas, VA) was used for all experiments. Strains were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.5% glycerol, 10% albumin-dextrose-catalase enrichment, and 0.05% Tween 80 at 37°C with constant shaking at 220 RPM in an orbital shaker. All drugs were obtained from the following commercial vendors: Toronto Research Chemicals (ertapenem) and Sigma-Aldrich (rifampin, meropenem, imipenem, doripenem, biapenem, faropenem, tebipenem, and all cephalosporins). To assess the quality of these compounds, meropenem, biapenem, and tebipenem were randomly selected and assessed by liquid chromatography-mass spectrometry. The purity of compounds ranged from 95% to 99%.

MIC. The MIC of each drug against *M. abscessus* was determined using the standard broth dilution method (50, 51) in accordance with CLSI guidelines specific for this organism (38). In summary, powdered drug stocks were reconstituted in dimethyl sulfoxide (DMSO), and 2-fold serial dilutions were prepared in Middlebrook 7H9 broth to obtain final drug concentrations ranging from 256 μg/ml to 1 μg/ml in 96-well plates in a final volume of 200 μl. A total of 10^5 CFU of *M. abscessus* from exponentially growing culture was added to each well. *M. abscessus* culture without drug and 7H9 broth alone were included in each plate as positive and negative controls, respectively. Plates were incubated at 30°C for 72 h per CLSI guidelines. MIC was assessed via visual inspection to determine growth or lack thereof, and an MIC for each drug was recorded as the lowest concentration at which *M. abscessus* growth was not observed. All MIC assessments were repeated to verify results.

Checkerboard titration assay. The checkerboard titration assay is a modified broth dilution assay and was performed as previously described (35–37). An initial synergy screen of two-drug combinations was performed at four different concentrations based on each drug's respective MIC: 2× MIC, MIC, one-half MIC, and one-fourth MIC for each drug in combination via 2-fold serial dilutions in a 96-well plate. A total of 10^5 CFU of *M. abscessus* from exponentially growing culture was inoculated into each

well, with positive and negative controls as described above for the MIC assay, and plates were incubated at 30°C for 72 h. Plates were visually inspected for *M. abscessus* growth or lack thereof. Drug combinations that inhibited *M. abscessus* growth at one-fourth MIC or less of each drug were considered to have some degree of synergy and were chosen for additional synergy testing.

To confirm the degree of synergy, two drugs were added to Middlebrook 7H9 broth in a 96-well plate, each starting at 2× MIC and serially diluted 2-fold up to 1/32× MIC, so all possible 2-fold dilution combinations from 2× to 1/32× MIC were assayed. A total of 10^5 CFU of *M. abscessus* was inoculated into each well. Plates were incubated at 30°C and evaluated for *M. abscessus* growth by visual inspection at 72 h. The fractional inhibitory concentration (FIC) of each drug in combination was determined as described previously (35–37). The FIC of a drug in a sample is calculated as the concentration of the drug divided by the MIC of the drug when used alone. The FIC Index (FICI) is the sum of the FIC of two drugs in a sample. The FICI was calculated for each combination of drugs that inhibited *M. abscessus* growth at less than one-half of the MIC of each individual drug. An FICI of ≤0.5 was interpreted as synergy, >0.5 to 2 as indifference, and >2 as antagonism. As an internal control, the MIC of each individual drug was also assessed via broth microdilution within each plate. All combinations with an FICI of ≤0.5 were tested in triplicate to confirm reproducibility, and an average FICI was calculated and reported here.

Determination of frequency of spontaneous drug resistance emergence. Any drug combination with an FICI of ≤0.5 was further assessed for frequency of spontaneous drug resistance. The CFU per milliliter of *M. abscessus* in culture at an A_{600} of 1.0 was initially determined as follows. *M. abscessus* was grown to exponential phase, adjusted to an A_{600} of 1.0 in Middlebrook 7H9 broth, and was serially diluted 10-fold in this broth. A total of 100 μl of each dilution was plated onto Middlebrook 7H10 agar, which was incubated at 37°C for 72 h. Resultant CFU counts were used to determine *M. abscessus* CFU density in culture. This assessment was repeated three times, and the mean *M. abscessus* CFU density was used in calculations in subsequent experiments.

To determine frequency of spontaneous drug resistance emergence, 10 ml of *M. abscessus* culture grown to exponential phase in 7H9 broth was used to prepare a suspension at an A_{600} of 1.0, and 1.0 ml of this suspension was inoculated onto each of 10 total Middlebrook 7H10 agar plates, which were supplemented with either a single drug or a combination of two drugs. The input *M. abscessus* was $\sim 1 \times 10^9$ CFU per plate. Therefore, the limit of detection of resistant mutant is $\sim 1 \times 10^{10}$. If we were unable to isolate a resistant CFU, we assigned a resistance frequency of $< 1 \times 10^{10}$. These assessments were performed at the MIC for single-drug and combination plates to promote selection of resistant mutants. CFU were counted after 7 days of incubation at 37°C. The frequency of drug-resistant mutants was determined from the number of spontaneous mutants observed as a percentage of the input CFU inoculum.

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