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SUSCEPTIBILITY

In Vitro Synergism of Rifabutin with Clarithromycin, Imipenem, and Tigecycline against the *Mycobacterium abscessus* Complex

Aristine Cheng,[a] Yi-Tzu Tsai,[a] Shu-Yuan Chang,[a] Hsin-Yun Sun,[a] Un-In Wu,[a] Wang-Huei Sheng,[a] Yee-Chun Chen,[a] Shan-Chwen Chang[a]

[a]Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

ABSTRACT Infections caused by the difficult-to-treat bacterium *Mycobacterium abscessus* are increasing in frequency. Rifabutin, in contrast to rifampin, appears to be active *in vitro* against *M. abscessus*, especially against clarithromycin-resistant strains. However, explorations for potential synergy between rifabutin and available antimicrobials are currently limited. *In vitro* synergism between rifabutin and 10 antimicrobials was evaluated in 31 mycobacterial strains by the checkerboard method. The fractional inhibitory concentration index (FICI) was calculated for each rifabutin-based combination. The colony morphology was recorded. Molecular methods for determination of the *M. abscessus* subspecies and analysis of macrolide resistance were performed by sequencing of the *secA1*, *rpoB*, *hsp65, erm*(41), and *rrl* genes. Rifabutin yielded an MIC_{50} of 16 mg/liter (range, 2 to 32 mg/liter) against 26 clinical *M. abscessus* isolates (comprising 13 *M. abscessus* subsp. *abscessus* and 13 *M. abscessus* subsp. *massiliense* isolates) and 5 reference strains, including *M. abscessus* subsp. *abscessus* ATCC 19977, *M. abscessus* subsp. *bolletii* BCRC 16915, *M. abscessus* subsp. *massiliense* BCRC 16916, *M. chelonae* ATCC 35752, and *M. peregrinum* ATCC 700686. Significant synergism, classified by an FICI of ≤0.5, was demonstrated for the combinations of rifabutin and imipenem in 100% of *M. abscessus* subsp. *abscessus* and 69% of *M. abscessus* subsp. *massiliense* isolates, and significant synergism for rifabutin and tigecycline was demonstrated in 77% of *M. abscessus* subsp. *abscessus* and 69% of *M. abscessus* subsp. *massiliense* isolates. Among the 6 clarithromycin-resistant (MICs ≥ 8 mg/liter) *M. abscessus* subsp. *abscessus* isolates, the combination of rifabutin and clarithromycin was 100% synergistic. Rifabutin showed promising *in vitro* synergism with first-line anti-*M. abscessus* agents, especially for macrolide-resistant *M. abscessus* subsp. *abscessus* isolates.

KEYWORDS *Mycobacterium abscessus*, imipenem, *in vitro* synergy, macrolide resistance, rifabutin

Mycobacterium abscessus is a notorious multidrug-resistant pathogen which has emerged as a global threat among chronic lung disease, surgical, and neutralizing anti-interferon gamma autoantibody-producing patients (1–4). In addition to causing challenging and possibly lifelong infections among individuals, it has also caused large outbreaks in health care settings due to resistance to antiseptics and disinfectants (5–7). *M. abscessus* is intrinsically resistant not only to the classical antituberculosis drugs but also to most currently available antimicrobials (8). Of 1,040 FDA-approved drugs screened, only 7 compounds demonstrated activity with an MIC of ≤8 mg/liter against *M. abscessus* (9).

It was therefore surprising that a recent study showed rifabutin to be active *in vitro* against *M. abscessus*, especially against clarithromycin-resistant strains (10). Although clarithromycin has become the drug of choice for *M. abscessus* infections and therapeutic successes were reported in the 1990s, clarithromycin resistance has since been

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Address correspondence to Shan-Chwen Chang, changsc@ntu.edu.tw.

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associated with primary and secondary treatment failures (11). In most *M. abscessus* subsp. *abscessus* isolates, a functional erythromycin ribosome methyltransferase gene [(*erm*)41] leads to intrinsic inducible macrolide resistance. In *M. abscessus* subsp. *massiliense*, however, truncation and, thus, a loss of function of the *erm*(41) gene often restores susceptibility to macrolides (12). Macrolide resistance could also be determined by the presence of a thymine rather than a cytosine nucleotide at position 28 in *erm*(41) (i.e., the T28 and not the C28 sequevar) and by point mutations (at positions A2058 and A2059) in a region of the *rrl* gene encoding the peptidyltransferase domain of the 23S rRNA (12–14). Any of the above-described mechanisms causing macrolide resistance renders cure of *M. abscessus* infections unlikely in individuals with cystic fibrosis or neutralizing anti-interferon gamma autoantibodies (3, 15).

The aims of this study were to evaluate the potential *in vitro* activity of rifabutin and the effect of combining rifabutin with agents currently recommended for treatment of infections caused by *M. abscessus* isolates, such as clarithromycin, imipenem, amikacin, and tigecycline. We also investigated the effect of combining rifabutin with agents with which less clinical experience has accumulated, such as clofazimine and ceftibuten, to explore alternative treatment regimens.

RESULTS

A total of 26 clinical isolates, which comprised 13 *M. abscessus* subsp. *abscessus* and 13 *M. abscessus* subsp. *massiliense* isolates, as determined by multilocus sequence analysis (MLSA), were included in this study. The isolates were sporadic, and phylogenetic analysis confirmed that the isolates were not clustered in an outbreak. The 26 *M. abscessus* clinical isolates had been obtained from the blood ($n = 4$), cerebrospinal fluid ($n = 2$), lymph nodes ($n = 3$), surgical wounds ($n = 5$), skin and soft tissue ($n = 8$), pleural fluid ($n = 1$), ascites ($n = 1$) and lung ($n = 2$) of 26 patients with active disease.

Morphotypes. Of the 13 *M. abscessus* subsp. *abscessus* isolates, 10 exhibited a smooth colony morphology and 3 exhibited rough morphotypes. Similarly, 9 of the 13 *M. abscessus* subsp. *massiliense* isolates exhibited smooth colonies and 4 exhibited rough colonies.

***erm*(41) and *rrl* partial gene sequencing.** Among the 13 *M. abscessus* subsp. *abscessus* isolates, there were 3 isolates harboring the C28 variant and 10 isolates harboring the T28 variant of the *erm*(41) gene, and no point mutations were found in the *rrl* gene. In contrast, all except 1 of the 13 *M. abscessus* subsp. *massiliense* isolates harbored a truncated *erm*(41) gene. The one *M. abscessus* subsp. *massiliense* isolate with the full *erm*(41) gene harbored the C28 variant. In addition, 2 *M. abscessus* subsp. *massiliense* isolates from 2 different patients harbored *rrl* mutations (one A2057G and one A2058G point mutation).

Antimicrobial susceptibility testing. The single- and dual-drug (rifabutin-based) susceptibility test results for the 11 antimicrobial agents and 10 combinations are shown in Tables 1 and 2, respectively.

Single-drug susceptibility testing. The clarithromycin MIC_{50} and MIC_{90} were 4 and 8 mg/liter, respectively, for the *M. abscessus* subsp. *abscessus* isolates and 0.25 and 2 mg/liter, respectively, for the *M. abscessus* subsp. *massiliense* isolates. Phenotypic macrolide resistance was observed, which is in line with the presence of an intact *erm*(41) or T28 sequevar for the former isolates and the *rrl* point A2058G mutation for the latter isolates. The proportion of clarithromycin-resistant isolates was 46.2% and 15.4% for the *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* isolates, respectively.

Apart from the clear differences in macrolide susceptibility between the *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* isolates, the MIC_{50}/MIC_{90} of amikacin, ceftibuten, doxycycline, moxifloxacin, and tigecycline tested alone for both subspecies were exactly the same at 32/32 mg/liter, 256/256 mg/liter, >64/>64 mg/liter, 32/32 mg/liter, and 0.5/1 mg/liter, respectively. There were also few intersubspecies differences in the MIC_{50} and MIC_{90} values for rifabutin, clofazimine, cefoxitin, and imipenem. In other words, all *M. abscessus* isolates, regardless of the subspecies, had

TABLE 1 MICs of 11 antimicrobials tested individually against *Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *massiliense* isolates[a]

Subspecies and drug	MIC (mg/liter)			Susceptibility breakpoint (mg/liter)[b]		
	50%	90%	Range	Susceptible	Intermediate	Resistant
M. abscessus subsp. *abscessus* ($n = 13$)[c]						
Rifabutin	16	32	8 to 32	**≤2** (0, 0)		**>2** (13, 100.0)
Clarithromycin	4	8	0.5 to 16	**≤2** (5, 38.5)	**4** (2, 15.4)	**≥8** (6, 46.2)
Amikacin	32	32	16 to 64	**≤16** (2, 15.4)	**32** (10, 76.9)	**≥64** (1, 7.7)
Cefoxitin	32	32	16 to 64	**≤16** (1, 7.7)	**32–64** (12, 92.3)	**≥128** (0, 0)
Ceftibuten	256	256	128 to 256	**≤16** (0, 0)	**32–64** (0, 0)	**≥128** (13, 100.0)
Clofazimine	1	2	0.5 to 2	**≤2** (13, 100.0)		**≥8** (0, 0)
Doxycycline	>64	>64	>64	**≤1** (0, 0)	**2-4** (0, 0)	**≥8** (13, 100.0)
Imipenem	16	16	8 to 64	**≤4** (0, 0)	**8–16** (11, 84.6)	**≥32** (2, 15.4)
Linezolid	32	32	4 to 64	**≤8** (1, 7.7)	**16** (0, 0)	**≥32** (12, 92.3)
Moxifloxacin	32	32	8 to 256	**≤1** (0, 0)	**2** (0, 0)	**≥4** (13, 100)
Tigecycline	0.5	1	0.25 to 1	**≤0.5** (10, 76.9)	**1** (3, 23.1)	**≥2** (0, 0)
M. abscessus subsp. *massiliense* ($n = 13$)[d]						
Rifabutin	16	16	2 to 16	**≤2** (1, 7.7)		**>2** (12, 92.3)
Clarithromycin	0.25	2	<0.03 to >256	**≤2** (11, 84.6)	**4** (0, 0)	**≥8** (2, 15.4)
Amikacin	32	32	8 to 64	**≤16** (3, 23.1)	**32** (9, 69.2)	**≥64** (1, 7.7)
Cefoxitin	32	64	16 to 128	**≤16** (2, 15.4)	**32-64** (10, 76.9)	**≥128** (1, 7.7)
Ceftibuten	256	256	128 to 256	**≤16** (0, 0)	**32-64** (0, 0)	**≥128** (13, 100.0)
Clofazimine	2	2	0.5 to 2	**≤2** (13, 100.0)		**≥8** (0, 0)
Doxycycline	>64	>64	8 to >64	**≤1** (0, 0)	**2-4** (0, 0)	**≥8** (13, 100.0)
Imipenem	16	16	8 to 64	**≤4** (0, 0)	**8–16** (11, 84.6)	**≥32** (2, 15.4)
Linezolid	32	32	1 to 32	**≤8** (2, 15.4)	**16** (4, 30.8)	**≥32** (7, 53.8)
Moxifloxacin	32	32	2 to 128	**≤1** (0, 0)	**2** (1, 7.7)	**≥4** (12, 92.3)
Tigecycline	0.5	1	0.06 to 1	**≤0.5** (10, 76.9)	**1** (3, 23.1)	**≥2** (0, 0)

[a]The results of broth microdilution susceptibility testing are for each antimicrobial tested alone.
[b]Data in parentheses represent the number, percent, of isolates with the indicated result. Data in bold indicate the MIC breakpoints applied in this study.
[c]There were T28 ($n = 10$) and C28 ($n = 3$) sequevars among the 13 isolates. None of the isolates had *rrl* mutations.
[d]A truncated *erm41* ($n = 12$), the C28 sequevar ($n = 1$), and *rrl* mutations (A2057G, A2058G; $n = 2$) were found among the 13 isolates.

clofazimine MICs of ≤2 mg/liter, and all except one *M. abscessus* subsp. *massiliense* isolate had a rifabutin MIC of >2 mg/liter. The majority of isolates were intermediately susceptible to cefoxitin (92.3% versus 76.9% for the *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* isolates, respectively) and imipenem (84.6% versus 84.6%), but linezolid resistance was greater among the *M. abscessus* subsp. *abscessus* isolates than among the *M. abscessus* subsp. *massiliense* isolates (92.9% versus 53.8%, respectively).

The colony morphology did not affect the susceptibility of the *M. abscessus* isolates to amikacin, cefoxitin, ceftibuten, doxycycline, and moxifloxacin (data not shown). However, lower MIC_{50}/MIC_{90} values for rough versus smooth morphotypes for clofazimine (0.5/1 versus 2/2 mg/liter), rifabutin (4/16 versus 16/32 mg/liter), linezolid (16/32 versus 32/64 mg/liter), and tigecycline (0.25/0.25 versus 0.5/1 mg/liter), in contrast to higher MIC_{50}/MIC_{90} values for imipenem (16/64 versus 8/16 mg/liter), were observed. The two rough colony-forming *M. abscessus* subsp. *abscessus* T28 variants had an unexpectedly low clarithromycin MIC of 1 mg/liter, whereas the other smooth colony-forming T28 variants were clarithromycin resistant, with clarithromycin MICs ranging from 4 to 16 mg/liter.

Dual-drug (rifabutin-based) susceptibility testing by the checkerboard method and FIC determination. Synergy could be demonstrated for the combinations of rifabutin and imipenem in 100% and 69.2% of *M. abscessus* subsp. *abscessus* isolates and *M. abscessus* subsp. *massiliense* isolates, respectively. Remarkably, in combination with rifabutin and when the result was read at the minimum fractional inhibitory concentration (FIC), the MIC_{50} and MIC_{90} of imipenem and the proportion of imipenem-nonsusceptible isolates decreased from 16 to 1 mg/liter, from 32 to 2 mg/liter, and from 100% to 7.7%, respectively, for *M. abscessus* subsp. *abscessus*. The reciprocal decrease in the MIC_{50} and MIC_{90} of rifabutin (in combination with imipenem and when the result was read at the minimum FIC) and the proportion of isolates with rifabutin MICs of

TABLE 2 MICs of 10 antimicrobials tested in combination with rifabutin using the checkerboard broth microdilution method against *Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *massiliense* isolates

Subspecies and drug	MIC (mg/liter)			Categorical MIC breakpoints (mg/liter)[a]			No. (%) of isolates for which the combination showed synergy (FICI ≤ 0.5)
	50%	90%	Range	Susceptible	Intermediate	Resistant	
M. abscessus subsp. *abscessus* ($n = 13$)[b]							
Rifabutin	4*	8*	1 to 32	**≤2** (4, 30.8)		**>2** (9, 69.2)	
Clarithromycin	0.5*	1*	0.25 to 1	**≤2** (13, 100.0)	**4** (0, 0)	**≥8** (0, 0)	8 (61.5)
Amikacin	8*	16	1 to 32*	**≤16** (12, 92.3)	**32** (1, 7.7)	**≥64** (0, 0)	7 (53.8)
Cefoxitin	32	32	2 to 32	**≤16** (6, 46.2)	**32-64** (7, 53.8)	**≥128** (0, 0)	4 (30.8)
Ceftibuten	64*	128	8 to 128	**≤16** (1, 7.7)	**32-64** (8, 61.5)	**≥128** (4, 30.8)	8 (61.5)
Clofazimine	1	2	0.5 to 2	**≤2** (13, 100.0)		**≥8** (0, 0)	0 (0)
Doxycycline	>64	>64	32 to >64	**≤1** (0, 0)	**2–4** (0, 0)	**≥8** (13, 100.0)	0 (0)
Imipenem	1*	2*	0.5 to 8	**≤4** (12, 92.3)	**8–16** (1, 7.7)	**≥32** (0, 0)	13 (100.0)
Linezolid	16	32	4 to 32	**≤8** (5, 38.5)	**16** (3, 23.1)	**≥32** (5, 38.5)	4 (30.8)
Moxifloxacin	32	32	8 to 256	**≤1** (0, 0)	**2** (0, 0)	**≥4** (13, 100)	1 (7.7)
Tigecycline	0.12*	0.25*	0.06 to 0.25	**≤0.5** (13, 100.0)	**1** (0, 0)	**≥2** (0, 0)	10 (76.9)
M. abscessus subsp. *massiliense* ($n = 13$)[c]							
Rifabutin	8	16	0.12 to 16	**≤2** (3, 23.1)		**>2** (10, 76.9)	
Clarithromycin	0.12	0.25*	<0.03 to >256	**≤2** (12, 92.3)	**4** (0, 0)	**≥8** (1, 7.7)	3 (23.1)
Amikacin	16	16	4 to 32	**≤16** (11, 84.6)	**32** (2, 15.4)	**≥64** (0, 0)	2 (15.4)
Cefoxitin	32	32	4 to 64	**≤16** (4, 30.8)	**32–64** (9, 69.2)	**≥128** (0, 0)	2 (15.4)
Ceftibuten	128	128	32 to 256	**≤16** (0, 0)	**32–64** (4, 30.8)	**≥128** (9, 69.2)	2 (15.4)
Clofazimine	2	2	0.5 to 2	**≤2** (13, 100.0)		**≥8** (0, 0)	0 (0)
Doxycycline	>64	>64	4 to >64	≤1 (0, 0)	**2-4** (1, 7.7)	**≥8** (12, 92.3)	0 (0)
Imipenem	2*	8	0.25 to 32	**≤4** (10, 76.9)	**8–16** (2, 15.4)	**≥32** (1, 7.7)	9 (69.2)
Linezolid	16	16	0.5 to 32	**≤8** (6, 46.2)	**16** (5, 38.5)	**≥32** (2, 15.4)	2 (15.4)
Moxifloxacin	32	32	2 to 128	**≤1** (0, 0)	**2** (1, 7.7)	**≥4** (12, 92.3)	0 (0)
Tigecycline	0.12*	0.25*	0.06 to 0.25	**≤0.5** (13, 100.0)	**1** (0, 0)	**≥2** (0, 0)	9 (69.2)

[a]Data in parentheses represent the number, percent, of isolates with the indicated result. Data in bold indicate the MIC breakpoints applied in this study. The results of the broth microdilution susceptibility testing for two-drug combinations read at the minimal fractional inhibitory concentration index (FICI) are marked by an asterisk (*) if a fourfold or more decrease was observed when the drug was tested in rifabutin-based combinations (as shown here) compared to when it was tested alone (Table 1). For the case of rifabutin, the two-drug combination results refer to those for rifabutin combined with clarithromycin.

[b]There were T28 ($n = 10$) and C28 ($n = 3$) sequevars among the 13 isolates. None of the isolates had *rrl* mutations.

[c]A truncated *erm41* ($n = 12$), the C28 sequevar ($n = 1$), and *rrl* mutations (A2057G, A2058G; $n = 2$) were found among the 13 isolates.

>2 mg/liter was from 16 to 1 mg/liter, from 32 to 2 mg/liter, and from 100% to 7.7%, respectively, for *M. abscessus* subsp. *abscessus*. The combination of rifabutin and imipenem was synergistic for all other standard strains of rapidly growing mycobacterial isolates, including *M. abscessus* subsp. *bolletii*, *M. chelonae*, and *M. peregrinum* (Table 3).

Synergy could be demonstrated for the combination of rifabutin and tigecycline in 76.9% of *M. abscessus* subsp. *abscessus* isolates and 69.2% of *M. abscessus* subsp. *massiliense* isolates. The combination of rifabutin and tigecycline lowered the MIC_{50} and MIC_{90} of tigecycline and the proportion of isolates with tigecycline MICs of >0.5 mg/liter from 0.5 to 0.12 mg/liter, from 1 to 0.25 mg/liter, and from 23.1% to 0%, respectively, for both subspecies of *M. abscessus*. The reciprocal decrease in the MIC_{50} and MIC_{90} of rifabutin (in combination with tigecycline) and the proportion of isolates with rifabutin MICs of >2 mg/liter was from 16 to 4 mg/liter, from 32 to 8 mg/liter, and from 93.3% to 61.5%, respectively, for both subspecies of *M. abscessus*. Stratified by intrinsic macrolide resistance, rifabutin-tigecycline synergy was observed in 3 of 5 (60%) clarithromycin-susceptible and in 7 of 8 (88%) of clarithromycin-nonsusceptible *M. abscessus* subsp. *abscessus* isolates. This combination was also synergistic for the reference strains of *M. abscessus* subsp. *bolletii* and *M. chelonae* but not for *M. peregrinum* (Table 3).

When clarithromycin was combined with rifabutin, synergy was seen in 61.5% ($n = 8$) of *M. abscessus* subsp. *abscessus* isolates and in 23.1% ($n = 3$) of *M. abscessus* subsp. *massiliense* isolates. Stratified by initial clarithromycin susceptibility, rifabutin-clarithromycin synergy was observed in 1 of 5 (20%) clarithromycin-susceptible and in 6 of 6 (100%) clarithromycin-resistant *M. abscessus* subsp. *abscessus* isolates and in 2 of

TABLE 3 Antimicrobial susceptibility testing of reference strains of rapidly growing mycobacteria by checkerboard broth microdilution method[a]

Mycobacterial strain	Rifabutin + clarithromycin					Rifabutin + amikacin					Rifabutin + cefoxitin					Rifabutin + ceftibuten				
	MIC (mg/liter)					MIC (mg/liter)					MIC (mg/liter)					MIC (mg/liter)				
	A	a	B	b	FICI	A	a	B	b	FICI	A	a	B	b	FICI	A	a	B	b	FICI
M. abscessus subsp. *abscessus* ATCC 19977	32	32	0.5	0.5	2	32	4	32	8	**0.38**	32	32	>256	>256	2	32	16	256	128	1
M. abscessus subsp. *bolletii* BCRC 16915	16	2	4	0.25	**0.19**	16	8	32	16	1	16	16	32	32	2	16	4	256	128	0.75
M. abscessus subsp. massiliense BCRC 16916	8	2	0.5	0.25	**0.75**	8	4	32	16	1	8	2	64	32	0.75	8	2	256	128	0.75
M. peregrinum ATCC 700686	16	8	0.5	0.25	1	16	4	1	0.25	**0.5**	16	4	16	2	**0.38**	8	8	256	256	2
M. chelonae ATCC 35752	16	16	0.25	0.25	2	8	2	64	16	**0.5**	16	16	>256	>256	2	16	16	256	256	2
M. chelonae (clinical)	32	32	1	2	3	32	4	64	16	**0.38**	32	32	>256	>256	2	32	32	256	256	2

[a]A, MIC of rifabutin (drug A) tested alone; a, MIC of rifabutin (drug A) tested with the second drug in the combination (drug B); B, MIC of the standard antimycobacterial drug B tested alone; b, MIC of the standard antimycobacterial drug B tested in combination with rifabutin; FICI, fractional inhibitory concentration index. FICIs of ≤0.5, indicating synergism, are in boldface.

11 (18%) clarithromycin-susceptible and in 1 of 2 (50%) clarithromycin-resistant *M. abscessus* subsp. *massiliense* isolates.

For *M. abscessus* subsp. *abscessus* isolates, the MIC_{50} and MIC_{90} for clarithromycin (when combined with rifabutin and when the result was read at the FIC index [FICI]) decreased from 4 to 0.5 mg/liter and from 8 to 0.5 mg/liter, respectively, and the proportion of clarithromycin-resistant isolates decreased from 61.5% to 0%. While for *M. abscessus* subsp. *massiliense* isolates the MIC_{50} and MIC_{90} for clarithromycin (in combination with rifabutin and when the result was read at the FICI) decreased from 0.25 to 0.12 mg/liter and from 2 to 0.25 mg/liter, respectively, and only one clarithromycin-resistant isolate remained. The reciprocal decrease in the MIC_{50} and MIC_{90} of rifabutin (in combination with clarithromycin) and the proportion of isolates with rifabutin MICs of >2 mg/liter was from 16 to 4 mg/liter, from 32 to 8 mg/liter, and from 100% to 69.2%, respectively, for the *M. abscessus* subsp. *abscessus* isolates. The MIC_{50} and MIC_{90} of rifabutin (in combination with clarithromycin) for *M. abscessus* subsp. *massiliense* isolates did not change much and went from 16 to 8 mg/liter and from 16 to 16 mg/liter, respectively, and the proportion of *M. abscessus* subsp. *massiliense* isolates with rifabutin MICs of >2 mg/liter from 92.3% to 76.9%.

The combination of rifabutin with doxycycline or with clofazimine was indifferent for all *M. abscessus* isolates. In fact, the MICs of the individual drugs for these mycobacteria remained the same when they were tested alone or in combination. However, the combination of rifabutin with doxycycline was synergistic for *M. chelonae* ATCC 35752 (Table 3).

The combination of rifabutin with amikacin, cefoxitin, ceftibuten, or linezolid was synergistic in ≤60% *M. abscessus* isolates, with more synergy being demonstrated for *M. abscessus* subsp. *abscessus* than for *M. abscessus* subsp. *massiliense*. Rifabutin with amikacin was synergistic for the *M. peregrinum* ATCC 700686 and *M. chelonae* ATCC 35752 isolates. Rifabutin with moxifloxacin was synergistic for only one *M. abscessus* subsp. *abscessus* strain and was indifferent for all other rapidly growing mycobacteria tested in this study. None of the tested combinations demonstrated antagonism (FICI > 4) for any isolates.

The colony morphology also appeared to affect *in vitro* synergism. Isolates with rough morphotypes demonstrated less synergism than isolates with smooth morphotypes with the combinations of rifabutin with clarithromycin (14.3% versus 62.5%), imipenem (57.1% versus 93.8%), tigecycline (28.6% versus 87.5%), and ceftibuten (28.5% versus 50.0%) but better synergism with the combinations of rifabutin with cefoxitin (71.4% versus 6.3%), linezolid (57.1% versus 12.5%), and amikacin (57.1% versus 31.2%).

TABLE 3 (Continued)

Rifabutin + clofazimine					Rifabutin + doxycycline					Rifabutin + linezolid					Rifabutin + moxifloxacin					Rifabutin + imipenem					Rifabutin + tigecycline				
MIC (mg/liter)					MIC (mg/liter)					MIC (mg/liter)					MIC (mg/liter)					MIC (mg/liter)					MIC (mg/liter)				
A	a	B	b	FICI	A	a	B	b	FICI	A	a	B	b	FICI	A	a	B	b	FICI	A	a	B	b	FICI	A	a	B	b	FICI
32	16	2	1	1	32	32	>64	>64	2	32	16	64	32	1	32	32	16	32	3	32	8	16	4	**0.5**	32	8	0.5	0.12	**0.49**
16	16	2	2	2	16	16	>64	>64	2	16	8	64	32	1	16	16	32	32	2	16	1	16	0.5	**0.09**	16	2	0.5	0.06	**0.25**
8	8	2	2	2	8	1	2	0.25	**0.25**	8	4	32	16	1	8	8	32	32	2	8	0.25	64	4	**0.09**	8	0.5	2	0.25	**0.19**
16	8	1	0.5	1	8	1	0.5	0.25	0.63	8	4	8	4	1	8	8	<0.25	<0.25	2	8	1	4	0.25	**0.19**	8	4	0.12	0.06	**1**
16	16	2	2	2	16	4	32	4	**0.38**	8	8	2	2	2	16	8	0.5	0.25	1	16	1	4	0.5	**0.19**	16	2	0.5	0.015	**0.16**
32	32	1	1	2	32	4	1	0.25	**0.38**	32	32	32	32	2	32	32	16	16	2	32	8	32	8	**0.5**	32	8	0.5	0.12	**0.49**

DISCUSSION

Patients with *M. abscessus* infections are routinely treated with clarithromycin along with two other antibiotics, usually amikacin, imipenem, or tigecycline (8, 15). The clinical utility of these antibiotic combinations is limited by the induction of resistance to clarithromycin and by their respective toxicities (16). Our present study confirmed that rifabutin holds promising activity against *M. abscessus*, and combinations comprising this drug with the core recommended treatment for clarithromycin-resistant *M. abscessus* are synergistic (10, 15).

We showed that for the *M. abscessus* subsp. *abscessus* isolates harboring inducible macrolide resistance, determined by the presence of an intact *erm*(41) with a thymine rather than a cytosine nucleotide at position 28, the combination of rifabutin and clarithromycin was reliably (100%) synergistic *in vitro*. For the highly macrolide-resistant *M. abscessus* subsp. *massiliense* isolate harboring the acquired A2058G point mutation in the *rrl* gene, the combination of rifabutin and clarithromycin was not significantly more active than either drug tested alone; however, for the phenotypically macrolide-resistant *M. abscessus* subsp. *massiliense* isolate with a truncated *erm*(41) gene and a wild-type *rrl* gene, the combination was significantly more active than either drug tested alone. Practically speaking, regardless of subspecies, if the initial clarithromycin MIC on the 3rd to 5th day was in the range of 0.5 to 16 mg/liter, the presence or absence of inducible macrolide resistance did not alter the synergism observed between rifabutin and clarithromycin. However, if the initial clarithromycin MIC was >256 mg/liter, point mutations in the *rrl* gene were more likely to underlie such an excessively high level of macrolide resistance and addition of rifabutin to clarithromycin was not considered synergistic and could no longer lower clarithromycin MICs to the susceptible range. If the *in vitro* synergism between rifabutin-clarithromycin is clinically validated, this may circumvent the need for extending incubation periods to 14 days for antimicrobial susceptibility testing or routine molecular determination of *erm*(41) and the *M. abscessus* subspecies.

Of note, although an FICI of ≤0.5 for clarithromycin-susceptible *M. abscessus* strains could not be achieved since it was difficult to determine a further 4-fold decrease in isolates with low baseline clarithromycin MICs (0.25 to <0.03 mg/liter), even for these isolates, a 4-fold or more synergistic decrease in the MICs of rifabutin in combination with clarithromycin could be demonstrated for most isolates (e.g., from 16 mg/liter to 2 mg/liter or from 8 mg/liter to 0.5 mg/liter). This lowered *in vitro* MIC of rifabutin would fall to concentrations that would be achievable in the lung (2 mg/liter) and in the serum (1 mg/liter) (17, 18).

In addition, imipenem and rifabutin were reliably synergistic for 100% of the *M. abscessus* subsp. *abscessus* isolates, for 69% of the *M. abscessus* subsp. *massiliense* isolates, and for the *M. abscessus* subsp. *bolletii*, BCRC 16915 isolate. The combination of tigecycline and rifabutin also demonstrated reliable synergism against 77% of the *M.*

abscessus subsp. *abscessus* isolates, against 69% of the *M. abscessus* subsp. *massiliense* isolates, and against the *M. abscessus* subsp. *bolletii* BCRC 16915 isolate.

Our results are supported by those of two smaller studies recently published online. Rifabutin-clarithromycin synergism was demonstrated in one study in which synergism (FICI $\leq$ 0.5) was demonstrated by the checkerboard method for 3 of 6 (50%) clinical isolates of *M. abscessus* (all of which were clarithromycin susceptible with an MIC of $\leq$2 mg/liter and not delineated to the subspecies level) alongside the reference *M. abscessus* ATCC 19977 strain, and additivity (FICI = 0.63 to 0.75) was demonstrated for the remaining 3 isolates (19). In the same study, rifabutin-tigecycline synergism with an FICI of $\leq$0.5 was also observed for 3 of the 6 (50%) clinical isolates, while additive effects with an FICI of 0.75 were shown for the remaining isolates (19). In another study, where only a single strain of *M. abscessus* (ATCC 19977) and its β-lactamase-deficient derivative were studied, the rifabutin-imipenem combination was also shown to be synergistic by both the checkerboard method and a time-kill curve assay (20).

However, more convincingly in that study, rifabutin-imipenem was more effectively synergistic and bactericidal in a macrophage model (20). A 9-fold intracellular accumulation of rifabutin in human polymorphonuclear leukocytes has been reported; hence, our modest *in vitro* effects may, if anything, underestimate the effects *in vivo* (21).

Taken together, the inclusion of rifabutin as a frontline partner in combination therapy against *M. abscessus* infections should be investigated in therapeutic trials. Since amikacin activity is antagonized by clarithromycin-induced resistance genes via the expression of *whiB7*, a global regulator of intrinsic resistance genes which causes the upregulation of *eis2* (which confers resistance to amikacin) (22) and which is also antagonized by tigecycline via yet uncharacterized mechanisms (23), the replacement of amikacin, an intravenous, potentially nephrotoxic and ototoxic agent, with rifabutin, an oral agent with better tolerability, intracellular accumulation, and reliable synergism *in vitro* with all three frontline therapies (clarithromycin, imipenem, and tigecycline across subspecies), seems to be rational, especially in the event of acquired macrolide resistance. The influence of subspecies and the mechanism underlying macrolide resistance on the extent of rifabutin-based synergism suggested by this study deserves further corroboration.

In addition, we found that the colony morphology, which can be routinely observed in clinical laboratories, influences the susceptibility of *M. abscessus* to selected antimicrobials and their combinations. Similar to previous findings, we showed that rough morphotypes had lower MICs to tigecycline (24) but higher MICs to imipenem than smooth morphotypes (25). We also showed that rough morphotypes were more susceptible to linezolid and rifabutin, which target the mycobacterial machinery involved in RNA and protein synthesis. This may be plausible, given that rough morphotypes correspond to cord-forming *M. abscessus* isolates associated with more invasive and persistent disease and smooth morphotypes correspond to non-cord-forming, biofilm-forming, and glycopeptidolipid-rich *M. abscessus* isolates (26–28). Putatively, therefore, rough morphotypes may require several redox proteins to resist oxidative attack from host macrophages due to their unmasking (29–31). Clofazimine also appears to be more active against rough morphotypes, possibly by interfering with the redox system, wherein it becomes reduced on interaction with NDH-2 and then subsequently oxidized in the presence of molecular oxygen, cycling between these two states and siphoning off electrons from the bacterial electron transport chain (32).

To the best of our knowledge, our investigation is the first to study rifabutin in combination with ceftibuten and linezolid, and these combinations were synergistic for a proportion of the *M. abscessus* isolates tested in our study. Given the requirement for prolonged therapy (6 to 12 months) for most cases of *M. abscessus* disease and the restricted availability of sophisticated molecular methods for determination of *M. abscessus* subspecies, the availability of oral combinatorial partners that can be applied based on easily made distinctions, such as colony morphology, is worth exploring. For example, the combination of rifabutin and ceftibuten was more favorably synergistic

for smooth morphotypes than for rough morphotypes (50% versus 29%), whereas for rifabutin and linezolid, the combination was more synergistic for rough morphotypes than for smooth morphotypes of *M. abscessus* (57% versus 12.5%).

In conclusion, remarkable synergy against *M. abscessus* was observed, in order of diminishing frequency, for rifabutin-based combinations with imipenem, tigecycline, clarithromycin, ceftibuten, amikacin, cefoxitin, and linezolid. Colony morphology, *rrl* point mutations, and subspecies identity but not inducible macrolide resistance impacted the rifabutin-based synergy. These findings merit clinical and mechanistic validation.

MATERIALS AND METHODS

Mycobacterial isolates. A total of 26 nonduplicate *M. abscessus* clinical isolates from 26 patients with skin and soft tissue, lymph node, pulmonary, central nervous system, and bloodstream infections at the National Taiwan University Hospital in Taipei, Taiwan, between January 2009 and December 2015 were studied. Isolates were submitted by clinicians to the research laboratory with a request for susceptibility testing. Five reference strains were also tested, including *M. abscessus* subsp. *abscessus* ATCC 19977, *M. abscessus* subsp. *bolletii* BCRC 16915, *M. abscessus* subsp. *massiliense* BCRC 16916, *M. peregrinum* ATCC 700686, *and M. chelonae* ATCC 35752. The smooth or rough morphology of these mycobacterial colonies was observed and recorded (26).

***secA1*, *rpoB*, *hsp65*, *erm*(41), and *rrl* partial gene sequencing.** Genomic DNA was extracted from mycobacterial cultures for identification and molecular typing based on the sequences of the genes *secA1*, *rpoB*, and *hsp65* (33). Details of the PCR method used were previously described by Zelazny et al. (34). Sequencing of *erm*(41) and *rrl* was performed using the methods described by Bastian et al. (14) and Maurer et al. (35). Sequences were analyzed and compared with those in the NCBI database using a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antimicrobial susceptibility testing. Broth microdilution in cation-adjusted Mueller-Hinton broth for rapidly growing mycobacteria, as suggested by the Clinical and Laboratory Standards Institute (CLSI), was used for drug susceptibility testing (36). The antimicrobial agents tested included rifabutin, clarithromycin, amikacin, cefoxitin, ceftibuten, doxycycline, imipenem, linezolid, moxifloxacin, clofazimine, and tigecycline. Susceptible, intermediately susceptible, and resistant breakpoints followed the CLSI guidelines for rapidly growing mycobacteria, with the exception of the breakpoints for (i) rifabutin, which were adopted from CLSI guidelines for *M. marinum* (36); (ii) tigecycline, which were adopted from a clinical study for *M. abscessus* (37), and (iii) clofazimine, which were adopted from a molecular resistance study for *M. abscessus* (38). For ceftibuten, an oral cephalosporin, no interpretative criteria for mycobacteria exist; hence, MIC breakpoints of $\leq$16, 32 to 64, and $\geq$128 mg/liter for susceptible, intermediately susceptible, and resistant, respectively, in line with the breakpoints for cefoxitin for descriptive purposes only, were used (36).

The MICs of the individual drugs were determined first. The checkerboard titration method was used to test the combination of rifabutin with another antimicrobial agent (39). Rifabutin was serially diluted along the ordinate, while the second drug was diluted along the abscissa. Concentrations ranging from 4 to 8 times the expected MIC to at least 1/8 to 1/16 the expected MIC were included to determine the occurrence and magnitude of synergism or antagonism. The MICs of the agents in combination were read between the 3rd and 5th day for all drugs if the control growth was positive. The fractional inhibitory concentration (FIC) was calculated using the formula $MIC_a/MIC_A + MIC_b/MIC_B$, where MIC_a represents the MIC of rifabutin (drug A) tested in combination, MIC_A represents the MIC of rifabutin tested alone, MIC_b represents the MIC of the other antimycobacterial drug (drug B) tested in combination, and MIC_B represents the MIC of other antimycobacterial drugs tested alone. The minimum FIC for each combination was defined as the FIC index (FICI). Synergy was defined as an FICI of $\leq$0.5, an FICI of between 0.5 and 4 was considered indifferent, and a FIC index of $>$4 indicated an antagonistic association (40). In addition, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains according to the CLSI M24-A2 guidelines (36).

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A.C. conceived of and designed the study, analyzed the results, and wrote the manuscript. Y.-T.T. conducted the experiments and analyzed the results. S.-Y.C. collected the mycobacterial isolates and helped execute the study. H.-Y.S. provided critical analysis and a review of the manuscript. U.-I.W. helped collect mycobacterial isolates and execute the study. W.-H.S. provided technical expertise and critiqued and reviewed the manuscript. Y.-C.C. coordinated the study and reviewed the manuscript. S.-C.C. provided technical expertise and critiqued and reviewed the manuscript.

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