In vitro evaluation of a new drug combination against clinical isolates belonging to the *Mycobacterium abscessus* complex

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Abstract

The *in vitro* susceptibility profile to amikacin, linezolid, clarithromycin, imipenem, cefoxitin, clofazimine and tigecycline was established for 67 strains belonging to the *Mycobacterium abscessus* complex. Clofazimine and tigecycline were among the most effective drugs, prompting us to assess the effect of a clofazimine and tigecycline combination. Synergistic activity was found in 42% of the 19 isolates tested. The clinical impact of this new drug combination against the *M. abscessus* complex, as an alternative or sequential medication for the treatment of drug-resistant strains, remains to be addressed.

Keywords: Clofazimine, cystic fibrosis, drug susceptibility, *Mycobacterium abscessus*, tigecycline
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Rapidly-growing mycobacteria (RGM) comprise species with increasing clinical importance, of which the *Mycobacterium abscessus* (*Mabs*) complex represents the most significant one [1]. This complex is subclassified into three subspecies, based on whole genome sequencing [2,3]: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* [4,5]. The distinction between these subspecies is clinically relevant because they respond differently to antibiotics [6]. Among RGM, the *Mabs* complex possesses the greatest capacity to colonize the respiratory tract and causes disease in patients with cystic fibrosis (CF) [7], associated with poor clinical outcome [8]. The importance of this complex in healthcare-associated diseases and in surgical tourism-associated infections also increases [9]. It comprises the most drug-resistant mycobacterial species [10] and treatments remain extremely challenging. Combination therapy that includes at least one effective aminoglycoside is strongly recommended for an effective treatment regimen [1]. However, few studies addressed the possible synergy of drug combinations against *Mabs* and given the lack of new active molecules, exploring the synergy between existing drugs represents a sensible way to achieve better treatment combinations.

Therefore, we first determined the drug susceptibility profile of a panel of *Mabs* complex isolates from CF and non-CF patients obtained between January 2008 and December 2013 at the Microbiology Laboratory, University Hospital, Montpellier, France. Species identification was performed using the GenoType Mycobacterium CM kit (Hain Lifescience, Nehren, Germany) and the multilocus sequence typing method (MLST) [11]. Most isolates were recovered from respiratory specimens. Among the 67 *M. abscessus* strains collected, 42 were subsp. *abscessus*, 21 subsp. *massiliense* and four subsp. *bolletii*. All were tested against amikacin, linezolid, clarithromycin, clofazimine (Sigma, Saint Quentin Fallavier, France), imipenem/cilastatin (Mylan, Saint-Priest, France), tigecyline (Pfizer, Amboise, Amboise, France) and cefoxitin (Panpharma, Fougères, France). MICs were determined according to the CLSI guidelines [12]. The broth microdilution method was used in Cation-Adjusted Mueller-Hinton Broth (CAMHB) with an inoculum of 5×10^5 CFU/mL in the exponential growth phase; 100 μL of drug dilutions were added to 100 μL of bacterial suspension and incubated at 30°C for 4 days. MICs were recorded by visual inspection and by absorbance at 560 nm to confirm visual recording. The categorization S/I/R for each antimicrobial was established according to the CLSI breakpoints [12].

MICs against the seven antimicrobials (Table 1) indicate that 87% of *Mabs* complex strains (58/67) were susceptible to amikacin whereas 12% (8/67) displayed intermediate susceptibility and 4% (3/67) were resistant. Eighty-four per cent of

TABLE 1. MICs (mg/L) of a panel of seven drugs in Cation-Adjusted Mueller-Hinton broth for 67 clinical isolates belonging to the *M. abscessus* complex

Drug	Clinical isolates (number of strains)	Number of strains with indicated MIC						MIC_{50}	MIC_{90}	Resistance (%)
		2	4	8	16	32	64			
Amikacin	All (67)	21	11	12	12	8	**3**	8	32	4
	M. abscessus (42)	12	9	5	8	6	**2**	4	32	5
	M. bolletii (4)	2		1	1			4	16	0
	M. massiliense (21)	7	2	6	3	2	**1**	8	32	5
	Smooth (50)	20	7	8	8	4	**3**	4	32	6
	Rough (17)	1	4	4	4	4		8	32	0
Linezolid	All (67)	30	14	14	4	**5**		4	16	7
	M. abscessus (42)	19	7	10	2	**4**		4	16	10
	M. bolletii (4)	3		1				2	8	0
	M. massiliense (21)	9	7	3	1	**1**		4	8	5
	Smooth (50)	27	6	11	3	**3**		2	16	6
	Rough (17)	3	8	3	1	**2**		4	32	12
Clarithromycin[a]	All (67)	32	13	**8**	6	7	1	2	16	12
	M. abscessus (42)	17	8	**7**	5	5		4	16	17
	M. bolletii (4)	4						2	2	0
	M. massiliense (21)	11	5	**1**	1	2	1	2	32	5
	Smooth (50)	30	7	**6**		3	4	2	32	12
	Rough (17)	2	6	**2**	3	3	1	8	32	12
Imipenem	All (67)		7	44	10	**6**		8	16	9
	M. abscessus (42)		5	25	6	**6**		8	32	14
	M. bolletii (4)		1	3				8	8	0
	M. massiliense (21)		1	16	4			8	8	0
	Smooth (50)		5	35	8	**2**		8	8	4
	Rough (17)		2	9	2	**4**		8	32	24
Cefoxitin	All (67)	16	12	17	13	7	**2**	8	32	3
	M. abscessus (42)	8	7	11	10	5	**1**	8	32	2
	M. bolletii (4)	3		1				2	2	0
	M. massiliense (21)	5	5	5	3	2	**1**	4	32	5
	Smooth (50)	15	7	13	9	4	**2**	8	32	4
	Rough (17)	1	5	4	4	3		8	32	0
Clofazimine	All (67)	53	8	6				2	4	
	M. abscessus (42)	34	4	4				2	4	
	M. bolletii (4)	3	1					2	4	
	M. massiliense (21)	16	3	2				2	4	
	Smooth (50)	38	7	5				2	4	
	Rough (17)	15	1	1				2	4	
Tigecycline	All (67)	20	21	18	8			4	8	
	M. abscessus (42)	10	15	13	4			4	8	
	M. bolletii (4)	2	1	1				2	8	
	M. massiliense (21)	8	5	5	3			4	16	
	Smooth (50)	17	15	12	6			4	8	
	Rough (17)	3	6	6	2			4	8	

[a]Data for clarithromycin are presented after 4 days only of incubation. Bold types indicate resistant categories (except to clofazimine and tigecycline) of interpretive criteria for each antimicrobial agent, according to the 2011 CLSI breakpoints. MIC_{50} and MIC_{90} are expressed as mean values of triplicates.

isolates (56/67) were susceptible and 7% (5/67) were resistant to linezolid. Clarithromycin proved to be the drug with the highest frequency of resistant strain (33%) (for a short duration of incubation), a proportion similar to the one reported previously [13]. MICs of imipenem show that 91% (61/67) of the *Mabs* complex strains display moderate susceptibility and 9% (6/67) were resistant to the drug. MIC_{50} and MIC_{90} were consistent with those reported recently [14]. Eighty-six per cent (58/67) of the isolates were susceptible to cefoxitin with a MIC_{90} value of 32 mg/L, slightly lower than the one reported earlier [14]. Regarding tigecycline, 61% (41/67) of all strains show MIC $\leq$ 4 mg/L, slightly lower than that reported previously [13]. Among all seven drugs, MIC_{50} and MIC_{90} values were found to be the lowest with clofazimine (2 and 4 mg/L, respectively).

Given the multiple and long antibiotic exposures experienced by CF patients, one may speculate that strains from CF patients exhibit higher antimicrobial resistance than those from non-CF patients, resulting in more resistant strains. Table S1 presents the drug susceptibility patterns of *Mabs* complex strains with (n = 35) and without underlying CF (n = 27). Only small variations were observed between the two groups with, at most, a two-fold difference in the MIC_{90} values, consistent with another study [13].

Clofazimine is currently registered for use in leprosy treatment and also to treat multidrug-resistant tuberculosis [15]. Two studies emphasized the synergistic activity of clofazimine when given with amikacin against *Mabs* [16,17]. Moreover, tigecycline given for more than 1 month as part of a multidrug regimen resulted in improvement in more than 60% of patients with *Mabs* and *M. chelonae* infections [18]. We thus explored the effect of clofazimine/tigecycline treatment against *Mabs* using the checkerboard microdilution technique in CAMHB. Susceptibility to clofazimine was determined at 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.031 mg/L, keeping tigecycline constant at 4 mg/L. Susceptibility to tigecycline

TABLE 2. MICs (mg/L) of clofazimine and tigecycline, alone or in combination against 19 *M. abscessus* complex isolates

Isolate	MIC of clofazimine		MIC of tigecycline		FIC[a] value/effect
	Singly	In combination	Singly	In combination	
#31 (*M.* abscessus)	8	4	4	0.03	0.5/Synergy
#35 (*M. abscessus*)	1	0.5	4	0.03	0.5/Synergy
#39 (*M. abscessus*)	8	4	4	0.03	0.5/Synergy
#40 (*M. abscessus*)	1	0.5	2	1	0.5/Synergy
#45 (*M. abscessus*)	4	2	2	0.03	0.5/Synergy
#54 (*M. abscessus*)	0.5	0.2	2	0.03	0.5/Synergy
#1 (*M. massiliense*)	1	0.03	8	0.06	0.3/Synergy
#8 (*M. massiliense*)	4	1	2	0.03	0.2/Synergy
#11 (*M. abscessus*)	0.5	1	2	0.12	2/Indifferent
#22 (*M. abscessus*)	2	2	8	0.12	1/Indifferent
#30 (*M. abscessus*)	0.5	0.5	2	0.03	1/Indifferent
#36 (*M. abscessus*)	1	2	2	0.03	2/Indifferent
#52 (*M. abscessus*)	1	2	2	0.03	2/Indifferent
#75 (*M. abscessus*)	2	2	8	0.03	1/Indifferent
#17 (*M. massiliense*)	2	0.03	2	2	1/Indifferent
#53 (*M. massiliense*)	2	4	8	0.03	2/Indifferent
#73 (*M. massiliense*)	8	4	8	8	1/Indifferent
#51 (*M. bolletii*)	2	0.03	2	2	1/Indifferent
#19 (*M. massiliense*)	1	4	16	0.125	4/Antagonistic

[a]The FIC index was calculated as the numerical sum of the two FICs for a given combination. Synergy was defined as an FIC index ≤0.5, indifference as an FIC index >1.0 to ≤2.0 and antagonism as an FIC index >2.

was assessed at 16, 8, 4, 2, 1, 0.5, 0.25 and 0.0625 mg/L with clofazimine at 1 mg/L. The effect was evaluated by calculating the fractional inhibitory concentration (FIC), defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug when used alone. Table 2 shows the MIC of clofazimine and tigecylcine, alone or in combination, against 19 clinical isolates. Synergism was found in 42% of the strains (8/19), and ten strains exhibited indifference, albeit the MIC for tigecycline was reduced when administered with clofazimine compared with that for tigecycline alone. One *M. massiliense* strain showed an antagonistic effect.

Activity of amikacin is considerably improved when combined with clofazimine [16,17]. Previous studies suggested that tigecycline does not have a good synergistic effect against RGM when combined with amikacin but displays high synergy with clarithromycin [19]. We report here the first combined activities of clofazimine and tigecycline with clear synergy in 42% of the strains tested. Although additional studies are required on a higher number of isolates, our data suggest that both drugs can be combined and used as an alternative or sequential medication for the treatment of drug-resistant strains or in difficult situations. However, synergy should be tested, rather than assumed, prior to treatment and care regarding the side-effects might also be taken into consideration. Efficient clofazimine analogues are also available, thus offering more alternatives in the long term. The clofazimine/tigecycline combination should next be assessed in animal models. In this context, we recently developed the zebrafish embryo infection model for *in vivo* drug assessment against *Mabs* [20].

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Transparency Declaration

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Susceptibility pattern of 62 *M. abscessus* complexes in CF (n = 35) and non-CF (n = 27) patients. The CF status was not known for five isolates.

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