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Original Article

In vitro activity of aminoglycosides, clofazimine, D-cycloserine and dapsone against 83 *Mycobacterium avium* complex clinical isolates

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Abstract *Background/Purpose:* Treatment success rates for *Mycobacterium avium* complex (MAC) diseases range from 50% to 55% only. To explore effective antimicrobials against either *Mycobacterium intracellulare* or *M. avium*, we determined *in vitro* activities of five aminoglycosides, clofazimine, dapsone and D-cycloserine compared with primary (clarithromycin) and secondary (moxifloxacin and linezolid) antimycobacterial agents.
Methods: 83 non-duplicate clinical MAC isolates were collected from sputum and identified at the species level by PCR and restriction enzyme analysis of the 65 kDa *hsp* and *rpoB* genes. Drug susceptibility testing was performed using broth microdilution method. The fractional inhibitory concentration was calculated to determine synergy between isepamicin and clofazimine.
Results: High susceptibility rates of five aminoglycosides (isepamicin, amikacin, kanamycin, streptomycin, capreomycin, 82.7–88%), D-cycloserine (82.7%), clofazimine (97.3%) and clarithromycin (92%) against *M. intracellulare*, and 2 aminoglycosides (isepamicin, streptomycin, 87.5%), D-cycloserine (100%) and clarithromycin (100%) against *M. avium* were found. Dapsone had no inhibitory activity and moxifloxacin had little effect against both *M. intracellulare* and

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M. avium. Linezolid had modest activity whereas clofazimine had little effect against *M. avium*. Most MAC isolates with non-susceptibility to isepamicin were also non-susceptible to the other four aminoglycosides. Most streptomycin-susceptible MAC isolates were also susceptible to amikacin. Synergistic effect of combination of isepamicin and clofazimine was demonstrated in all (100%) *M. intracellulare* isolates whereas in only 50% *M. avium* isolates.
Conclusion: When treating MAC diseases, species identification plays an important role in choosing treatment regimens. Combination of isepamicin and clofazimine may be a promising regimen in *M. intracellulare*-associated disease.
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Introduction

Most non-tuberculous mycobacteria (NTM) diseases are caused by *Mycobacterium avium* complex (MAC).[1] The current recommended combination regimen for MAC diseases includes macrolides (clarithromycin or azithromycin), rifamycins (rifampicin or rifabutin) and ethambutol. In cases with cavitary lesions and advanced or previously treated disease, injectable aminoglycosides (streptomycin or amikacin) should be considered.[1,2] However, the reported treatment success rate is still only 50%–55%.[3,4] Therefore, determining alternative effective antimicrobials against MAC is crucial.

Although there is ongoing debate about the role of *in vitro* susceptibility testing for the management of patients with MAC diseases and lacking of sufficient clinical trials's data demonstrating suitable drug choices,[1] *in vitro* susceptibility testing is still a reliable way to explore alternative effective antimicrobials against MAC. Several antimicrobials that are not currently recommended have been studied with regards to the *in vitro* inhibitory activity against MAC. Isepamicin has been reported to be the most potent aminoglycoside against MAC isolates.[5] In contrast, both kanamycin and capreomycin have been shown to be ineffective against *M. avium*.[6,7] Dapsone alone has been reported to have little activity against MAC isolates,[8] and studies on clofazimine and D-cycloserine have reported conflicting results.[9–11] However, most of these reports have only included a small number of MAC isolates and not identified to species level, thereby limiting their clinical application.

With the aim of exploring alternative effective antimicrobials against diseases caused by either *Mycobacterium intracellulare* or *M. avium*, we determined *in vitro* inhibitory activity of five aminoglycosides (isepamicin, amikacin, kanamycin, streptomycin, and capreomycin), clofazimine, D-cycloserine and dapsone compared with primary (clarithromycin) and secondary (moxifloxacin and linezolid) antimycobacterial agents against 83 clinical MAC isolates and combination effect of isepamicin and clofazimine.

Methods

Bacterial isolates

Eighty-three non-duplicate clinical MAC isolates were all collected from sputum in the mycobacterial laboratory in Taichung Veterans General Hospital since November 2011 to July 2013. These isolates were identified at the species level by polymerase chain reaction and restriction enzyme analysis of the 65 kDa *hsp* and *rpoB* genes.[12,13] *M. avium* ATCC 700898 was used as the control for the drug susceptibility tests. The Institutional Review Board and Ethics Committee of Taichung Veterans General Hospital approved this study.

Antimicrobial agents

Isepamicin was donated by TTY Biopharm Co. Ltd. (Taipei, Taiwan). Amikacin, kanamycin, streptomycin, capreomycin, and clarithromycin were purchased from U.S. Pharmacopeial Convention, Inc. (Rockville, USA). Clofazimine, D-cycloserine, dapsone, moxifloxacin and linezolid were purchased from Sigma Chemical Co. (St. Louis, MO).

Drug susceptibility testing

We performed drug susceptibility testing using broth microdilution for the MAC isolates.[14–16] Briefly, the 83 clinical MAC isolates and *M. avium* ATCC 700898 (as the control) were sub-cultured on 7H11 agar plates and incubated at 37 °C under 5% CO_2 for two weeks. Mycobacterial suspensions were prepared by scraping the confluent portion of growth on the agar plate into 1 mL of cation-supplemented Mueller-Hinton broth (CSMHB; Becton Dickinson Co.) broth containing 5% oleic acid, albumin, dextrose, and catalase. The suspensions were adjusted to McFarland 0.5 followed by serial 10-fold dilutions with CSMHB broth. The two (tested and control strains) mycobacterial suspension dilutions were then inoculated into the microdilution plates. Serial double dilutions of the tested antimicrobial agents were prepared with concentrations in the wells of culture test plates (MIDSCI, St. Louis, USA) ranging from 0.25 μg/mL to 128 μg/mL.

The inoculated trays were incubated at 37 °C in ambient air, and the results were interpreted after 7 days. If growth was poor, the trays were re-inoculated and re-read after 14 days according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M24-A2, Second Edition, 2011).[14] For primary (Clarithromycin) and secondary (moxifloxacin and linezolid) antimycobacterial agents, the interpretation of minimal inhibitory concentrations (MICs) was according to the CLSI guidelines (M24-A2, Second Edition, 2011).[14] For

the aminoglycosides, clofazimine, D-cycloserine and dapsone, no reference MICs data exist for MAC. Therefore, the interpretation of MICs of the aminoglycosides was tentatively made based on the breakpoints of amikacin for rapidly-growing mycobacteria.[16] Consequently, for the tested aminoglycosides, a MIC of ≤16 μg/mL was defined as being susceptible, 32 μg/mL as having intermediate susceptibility, and ≥64 μg/mL as being resistant. To determine the MIC breakpoints of clofazimine, D-cycloserine and dapsone, we tentatively grouped the MICs into three categories (susceptible, intermediate and resistant): ≤1 μg/mL, 2 μg/mL and ≥4 μg/mL for clofazimine, and ≤16 μg/mL, 32 μg/mL and ≥64 μg/mL for D-cycloserine and dapsone by referring to the MICs the breakpoints of clofazimine and D-cycloserine for *Mycobacterium kansasii* and *Mycobacterium tuberculosis* (but no MICs reference for dapsone are available for mycobacteria).[16,17] The reference strain *M. avium* ATCC 700898 was used as the control, with acceptable ranges of MICs recommended by the CLSI guideline (M100, Second Edition, 2011)[14] for *M. avium* ATCC 700898, i.e. 2—16 μg/mL for amikacin, 1.25—10 μg/mL for kanamycin, 4—32 μg/mL for streptomycin and 5—40 μg/mL for capreomycin (Thermo Fisher™ Sensititre SLOMYCOI, UK). In this study, all of the quality control results were within acceptable ranges.

Testing of the synergistic effect between isepamicin and clofazimine

The criteria of MAC isolates selected for synergistic tests was both non-susceptible to at least one of the tested five aminoglycosides and clofazimine MIC ≥0.5 μg/mL. For each isolate tested, 100 μL of 0.5× MIC of isepamicin in CSMHB was first added to a 96-well plate (MIDSCI). Serial doubling dilutions of clofazimine were then prepared with CSMHB, and the mycobacterial suspension in CSMHB was added to the drug dilutions to make a final concentration of 5×10^5 CFU/mL. Subsequently, 100 μL of this drug/mycobacteria mixture was added to the wells containing 100 μL of the isepamicin solution. The final concentration of isepamicin in the wells was 0.25× the original MIC, whereas the concentrations of clofazimine ranged from 0.0625 mg/L to 128 mg/L. If the MIC of clofazimine was <0.0625 mg/L, another plate with clofazimine concentrations ranging from 0.0078 mg/L to 16 mg/L was tested. The fractional inhibitory concentration (FIC) was calculated using the formula FIC = (MICa combination/MICa alone) + (MICb combination/MICb alone), where a and b stand for isepamicin and clofazimine, respectively. An FIC of ≤0.5 was interpreted as synergism, >0.5—4 as indifference, and >4 as antagonism.[18,19]

Results

A total of 83 MAC isolates were collected, of which 75 were identified as *M. intracellulare* and eight as *M. avium*. Eleven antimicrobial agents were tested for the antimicrobial activity against the 83 MAC isolates (Table 1). The five tested aminoglycosides had the same MIC range, MIC_{50}, and MIC_{90} (2—64 μg/mL, 8 μg/mL, and 32 μg/mL, respectively) against *M. intracellulare*, and the same MIC_{50} against *M. avium* but differences in both MIC range and MIC_{90}. The susceptibility rates of *M. intracellulare* isolates were 82.7—88% for all five tested aminoglycosides. However, *M. avium* isolates were more resistant to amikacin, kanamycin and capreomycin compared to isepamicin and streptomycin. D-cycloserine had high inhibitory activity against both *M. intracellulare* and *M. avium* (82.7% and 100%, respectively), whereas dapsone had no inhibitory activity against both species. Clofazimine had higher activity to most *M. intracellulare* isolates (97.3%) than *M. avium* isolates (12.5%) (Table 1). For primary (Clarithromycin) and secondary (moxifloxacin and linezolid) antimycobacterial agents, clarithromycin had high inhibitory activity against both *M. intracellulare* and *M. avium* (92% and 100%, respectively), whereas moxifloxacin had poor inhibitory activity against both species. Linezolid had higher activity against *M. avium* isolates (75%) than *M. intracellulare* isolates (6.7%) (Table 1).

Of the 83 tested MAC isolates, 19 (15 *M. intracellulare* isolates and 4 *M. avium* isolates) were non-susceptible (MIC ≥ 32 μg/mL) to at least one of the five tested aminoglycosides (Table 2). All 19 MAC isolates were susceptible to clarithromycin, but most isolates were non-susceptible to moxifloxacin and linezolid (Table 2). Of the 19 MAC isolates, 13.3% (2/15) of the *M. intracellulare* isolates were susceptible and 50% (2/4) of the *M. avium* isolates were intermediate to linezolid; 6.7% (1/15) of the *M. intracellulare* isolates was susceptible and 13.3% (2/15) of them were intermediate to moxifloxacin; 75% (3/4) of the *M. avium* isolates were intermediate to moxifloxacin.

Of the 19 MAC isolates, 20% (3/15) of the *M. intracellulare* isolates but none of the *M. avium* isolates had cross-resistance among the five tested aminoglycosides (MIC = 64 μg/mL to at least two of the five tested aminoglycosides). Sixty percent (9/15) of the *M. intracellulare* isolates and 25% (1/4) of the *M. avium* isolates were non-susceptible to all five aminoglycosides, and 13.3% (2/15) of the *M. intracellulare* isolates but none of the *M. avium* isolates were resistant to all of the five aminoglycosides. These findings indicate that the *M. intracellulare* isolates had a higher percentage of non-susceptibility and cross-resistance to the five aminoglycosides compared to the *M. avium* isolates. Clofazimine demonstrated good activity against *M. intracellulare* isolates that were non-susceptible to all five aminoglycosides (Table 2). However, 2.7% (2/75) of the *M. intracellulare* isolates (strain no. MAC81 and 82) with non-susceptible to all five aminoglycosides developed high MICs (MIC ≥ 4 μg/mL) to clofazimine (Table 2).

Two *M. intracellulare* isolates (strain no. MAC46 and MAC58) were resistant to isepamicin and also resistant to the other four tested aminoglycosides. Of 11 *M. intracellulare* isolates and one *M. avium* isolate that were non-susceptible to isepamicin, 81.8% (9/11) of the *M. intracellulare* isolates (strain no. V22, V67, V109, V159, V189, MAC46, MAC58, MAC81, and MAC82) and 100% (1/1) of the *M. avium* isolates (strain no. K282) were also non-susceptible to the other four tested aminoglycosides (Table 2). These findings indicate that isepamicin non-susceptibility could be a surrogate of aminoglycoside activity against both *M. intracellulare* and *M. avium* isolates.

All (64/64) of the streptomycin-susceptible *M. intracellulare* isolates, including strain no. V137, V172, MAC48

Table 1 *In vitro* activity of the tested antimicrobials against *Mycobacterium avium* complex clinical isolates.

Antimicrobial agent	MIC (μg/L)			Percent of isolates (%)		
	Range	50%	90%	S[a]	I[a]	R[a]
***M. intracellulare* (n = 75)**						
Isepamicin	2–64	8	32	85.3 (64/75)	12 (9/75)	2.7 (2/75)
Amikacin	2–64	8	32	88 (66/75)	9.3 (7/75)	2.7 (2/75)
Kanamycin	2–64	8	32	82.7 (62/75)	13.3 (10/75)	4 (3/75)
Streptomycin	2–64	8	32	85.3 (64/75)	9.3 (7/75)	5.3 (4/75)
Capreomycin	2–64	8	32	85.3 (64/75)	12 (9/75)	2.7 (2/75)
D-cycloserine	4–64	16	32	82.7 (62/75)	13.3 (10/75)	4 (3/75)
Dapsone	≥128	>128	>128	0 (0/75)	0 (0/75)	100 (75/75)
Clofazimine	≤0.25–8	≤0.25	1	97.3 (73/75)	0 (0/75)	2.7 (2/75)
Clarithromycin	<0.06–64	2	8	92 (69/75)	8 (6/75)	0 (0/75)
Linezolid	4–64	32	>64	6.7 (5/75)	12 (9/75)	81.3 (61/75)
Moxifloxacin	0.5–8	4	8	4 (3/75)	5.3 (4/75)	90.7 (68/75)
***M. avium* (n = 8)**						
Isepamicin	2–32	16	16	87.5 (7/8)	12.5 (1/8)	0 (0/8)
Amikacin	4–32	16	32	75 (6/8)	25 (2/8)	0 (0/8)
Kanamycin	2–32	16	32	75 (6/8)	25 (2/8)	0 (0/8)
Streptomycin	2–32	16	16	87.5 (7/8)	12.5 (1/8)	0 (0/8)
Capreomycin	4–64	16	64	50 (4/8)	25 (2/8)	25 (2/8)
D-cycloserine	4 to >16	8	16	100 (8/8)	0 (0/8)	0 (0/8)
Dapsone	>128	>128	>128	0 (0/8)	0 (0/8)	100 (8/8)
Clofazimine	1–4	4	4	12.5 (1/8)	25 (2/8)	62.5% (5/8)
Clarithromycin	2–8	4	8	100 (8/8)	0 (0/8)	0 (0/8)
Linezolid	16–64	32	64	75 (6/8)	25 (2/8)	0 (0/8)
Moxifloxacin	2–4	2	2	0 (0/8)	50 (4/8)	50 (4/8)

[a] Definition of susceptible, intermediate and resistant of tested antimicrobials.
Abbreviations: MIC, minimal inhibitory concentration. S, susceptible. I, intermediate. R, resistant.
Isepamicin, Amikacin, Kanamycin, Streptomycin, Capreomycin, D-cycloserine, Dapsone: S: ≤16 μg/mL, I: 32 μg/mL, R: ≥64 μg/mL.
Clarithromycin, Linezolid: S: ≤8 μg/mL, I: 16 μg/mL, R: ≥32 μg/mL.
Moxifloxacin, Clofazimine: S: ≤1 μg/mL, I: 2 μg/mL, R: ≥4 μg/mL.

and MAC59, were also susceptible to amikacin, and 75% (6/8) of the streptomycin-susceptible *M. avium* isolates, including strain no. N49 and U58, were also susceptible to amikacin. This indicates that being susceptible to streptomycin could be a surrogate of amikacin activity against both *M. intracellulare* and *M. avium* isolates (Table 2).

To determine the synergy between the tested drugs against MAC diseases, the effect of combining isepamicin and clofazimine was evaluated. Eight *M. intracellulare* isolates and four *M. avium* isolates were found to be both non-susceptible to at least one of the tested five aminoglycosides and clofazimine MIC ≥0.5 μg/mL. These isolates were then further tested for the synergy between isepamicin and clofazimine, and 100% (8/8) of the *M. intracellulare* isolates demonstrated synergy, with 4-fold decreases of MICs to clofazimine and 4- to 16-fold decreases of MICs to isepamicin, compared to only 50% (2/4) of the *M. avium* isolates (Table 3).

Discussion

Previous studies have demonstrated that isepamicin and streptomycin are the most potent aminoglycosides against MAC isolates compared to amikacin and kanamycin.[5,9] In our study, we found that all of the five tested aminoglycosides had good inhibitory activity against *M. intracellulare*. However, isepamicin and streptomycin were more potent aminoglycosides than amikacin, kanamycin and capreomycin against *M. avium*. This suggested that isepamicin is as effective as amikacin or streptomycin against both *M. intracellulare* and *M. avium*. However, the toxicity of aminoglycosides is a major concern in clinical practice. A previous clinical trial demonstrated that toxicity was common when treating pulmonary NTM diseases with inhaled amikacin compared to amikacin infusions.[20] In one previous study regarding the safety of isepamicin in adults,[21] severe or life-threatening adverse events, renal function impairment and ototoxicity were lower in patients receiving isepamicin compared to those receiving amikacin treatment. Michel Tod reported that isepamicin is less nephro-, vestibulo-, oto-toxicity as well as neuromuscular blocking activity than other aminoglycosides.[22] Considering its lower toxicity,[21,22] isepamicin may be considered as an alternative to other aminoglycoside-containing regimens for the treatment of MAC diseases.

One clinical trial reported that kanamycin was effective for the treatment of patients with MAC diseases. However, species identification of the MAC isolates from the participants was not disclosed.[23] Consistent with previous studies, we found that both kanamycin and capreomycin had poorer activity against *M. avium* than *M. intracellulare*.[6,7] These

Table 2 The distributions of tentative non-susceptibility to tested aminoglycosides and the corresponding clofazimine, clarithromycin, linezolid, moxifloxacin MICs.

Strain	MIC (μg/mL)								
	ISE	AMI	KAN	SM	CAP	CLO	CLA	LZD	MXF
***M. intracellulare* (n = 15)**									
V105	16	16	16	32	16	<0.25	8	64	8
MAC43	16	16	16	32	16	<0.25	4	64	4
V137	16	16	32	16	16	<0.25	1	8	4
MAC59	16	16	32	16	16	1	2	4	2
MAC48	32	16	32	16	32	0.5	4	>64	8
V172	32	16	32	16	32	0.5	0.25	32	1
V22[a]	32	32	32	32	32	<0.25	2	32	>8
V67[a]	32	32	32	32	32	<0.25	4	32	8
V109[a]	32	32	32	32	32	<0.25	2	64	2
V159[a]	32	32	32	32	32	0.5	2	64	4
V189[a]	32	32	32	32	32	0.5	4	>64	8
MAC81[a,d]	32	32	32	64	32	8	2	32	4
MAC82[a,b,d]	32	32	64	64	32	4	4	32	8
MAC46[a,b,c]	64	64	64	64	64	<0.25	4	>64	8
MAC58[a,b,c]	64	64	64	64	64	1	4	>64	4
***M. avium* (n = 4)**									
N49	8	16	16	16	32	1	4	16	4
U58	16	16	16	16	32	4	2	64	2
P28[d]	16	32	32	16	64	4	8	16	2
K282[a,d]	32	32	32	32	64	4	4	32	2

[a] Non-susceptible (MIC ≥ 32 μg/mL) to all five aminoglycosides.
[b] Cross-resistance among the five tested aminoglycosides. (MIC = 64 μg/mL to at least two of the five tested aminoglycosides).
[c] Resistant (MIC = 64 μg/mL) to all five aminoglycosides.
[d] Cross resistance between any tested aminoglycoside (MIC = 64 μg/mL) and clofazimine (MIC ≥ 4 μg/mL).
Abbreviations: MIC, minimal inhibitory concentration, ISE:Isepamicin, AMI:Amikacin, KAN:Kanamycin, SM:Streptomycin, CAP:Capreomycin, CLO:Clofazimine, CLA:Clarithromycin, LZD:Linezolid, MXF:Moxifloxacin.

Table 3 Synergy between isepamicin and clofazimine against eight selected *Mycobacterium intracellulare* and four *Mycobacterium avium* isolates.

Strain	MIC (mg/L)		In combination			
	Clofazimine	Isepamicin	Clofazimine	Isepamicin	FIC	Relationship
***M. intracellulare* (n = 8)**						
MAC48	0.5	32	0.125	4	0.375	Synergism
MAC58	1	64	0.25	4	0.3125	Synergism
MAC59	1	16	0.25	4	0.5	Synergism
MAC81	8	32	2	8	0.5	Synergism
MAC82	4	32	1	8	0.5	Synergism
V159	0.5	32	0.125	8	0.5	Synergism
V172	0.5	32	0.125	8	0.5	Synergism
V189	0.5	32	0.125	8	0.5	Synergism
***M. avium* (n = 4)**						
K282	4	32	1	8	0.5	Synergism
P28	4	16	1	8	0.75	Indifference
N49	1	8	0.25	8	1.25	Indifference
U58	4	16	1	4	0.5	Synergism

Abbreviations: MIC, minimal inhibitory concentration; FIC, fractional inhibitory concentration.

results indicate that both kanamycin and capreomycin may serve as an alternative treatment for *M. intracellulare*-associated infections rather than *M. avium*-associated infections. The aminoglycosides inhibit protein synthesis by interfering tRNA binding to the A site of ribosome and mRNA decoding.[24] The possible mechanism of resistance to aminoglycosides in mycobacteria is caused by mutations in either the 16S rRNA gene or the *rpsL* gene, leading to

modification of the 30S subunit of the ribosome or by producing acetyltransferase enzymes that inactive aminoglycosides.[25]

In our study, D-cycloserine had high inhibitory activity (<=16 μg/mL) against both *M. intracellulare* (82.7%) and *M. avium* (100%). Consistent with a previous study,[10] *M. avium* strains (100%) were more susceptible to cycloserine than *M. intracellulare* strains (82.7%). Rastogi et al. reported that D-cycloserine showed marginal activity (<=8 μg/mL) against MAC isolates.[9] This inconsistent result was due to MIC breakpoints selection. If 8 μg/mL was selected as MIC breakpoints of D-cycloserine in our study, only 25 (33.3%) *M. intracellulare* and 5 (62.5%) *M. avium* isolates (data not shown) were susceptible to cycloserine. However, only 10 MAC isolates were collected in Rastogi et al.'s study and not identified to the species level. Therefore, D-cycloserine was suitable to treat both *M. intracellulare*- and *M. avium*-associated diseases. D-cycloserine competitively inhibits two enzymes, alanine racemase (encoded by *alr* gene) and D-alanine ligase (encoded by *ddlA* gene) which are essential for peptidoglycan synthesis in the alanine metabolism pathway.[26,27] The mechanism of resistance to D-cycloserine is unknown in MAC but overexpression of *alr* and *ddlA* genes have been reported to confer resistance to D-cycloserine in *Mycobacterium smegmatis*. Recently, one study[28] showed that loss-of-function in *ald* gene (encoding L-alanine dehydrogenase) and mutations in *ald* and *alr* genes conferred resistance to D-cycloserine in *M. tuberculosis*.

We found that dapsone had no activity against either *M. intracellulare* or *M. avium* isolates, which is consistent with the finding in one previous study.[8] Rastogi et al. reported that the poor activity of dapsone against MAC was enhanced when used in combination with other drugs (ex: ethambutol and ethionamide) specifically acting at the mycobacterial cell-wall level.[8] This suggested that, for the treatment of MAC diseases, dapsone should be considered only in combination regimens. Dapsone (diaminodiphenylsulfone), an analogue of sulfonamides, is a inhibitor of dihydropteroate synthase (DHPS), an enzyme in the folate synthetic pathway. The mechanism of resistance to dapsone in mycobacteria has not been elucidated clearly and may be associated with mutations in the *folP1* or the *sull* genes, leading to the resistance of the DHPS to dapsone.[25]

Clofazimine has been reported to possibly be effective in patients with MAC diseases in clinical trials.[10,29,30] Consistent with these studies, we found that clofazimine had excellent activity against *M. intracellulare* isolates, including those non-susceptible to at least one of the five tested aminoglycosides, but little activity against *M. avium*. However, in those studies where the MAC isolates were not identified to the species level, the inhibitory activity of clofazimine against the MAC isolates exhibited conflicting results.[9,11] Therefore, clofazimine may be suitable to treat *M. intracellulare*-associated diseases, especially with aminoglycoside non-susceptibility, instead of *M. avium*-associated diseases. However, of note, 2.7% (2/75) of the *M. intracellulare* isolates with high MICs (MICs $\geq$ 4 μg/mL) to clofazimine were non-susceptible to all five tested aminoglycosides. The antimycobacterial activity of clofazimine was attributable to its high lipophilicity, enabling efficient transmembrane penetration and intracellular redox cycling. Clofazimine inhibit mycobacterial respiratory chain by competing for electrons with menaquinone, the substrate for type 2 NADH:quinone oxidoreductase.[31] Currently, no clofazimine-resistant mycobacterial clinical isolate has been reported. The *rv0678* gene mutation, encoding the Rv0678 protein drug efflux pump was described as the mechanism of clofazimine resistance in some *M. tuberculosis* studies.[32–34]

Cross-resistance among aminoglycosides has been demonstrated in *M. tuberculosis* and rapidly-growing mycobacteria.[35–37] In our study, among the MAC isolates exhibiting non-susceptibility to at least one of the five tested aminoglycosides, *M. intracellulare* isolates had the highest percentages of cross-resistance, non-susceptibility and resistance compared to the *M. avium* isolates. The reason for this distinction remains unknown, and further studies are warranted to elucidate this issue.

We found that isepamicin non-susceptibility could be a surrogate of aminoglycoside activity against both *M. intracellulare* and *M. avium* isolates. We also found that being sensitive to streptomycin could be a surrogate of amikacin activity against both *M. intracellulare* and *M. avium* isolates. These findings suggest that aminoglycosides should not be considered in combination regimens for MAC treatment if isepamicin MIC $\geq$32 μg/mL. On the other hand, both streptomycin and amikacin can be considered to be suitable antimicrobials against MAC if streptomycin MIC $\leq$16 μg/mL.

The synergy between clofazimine and amikacin has previously been demonstrated in both rapidly-growing and slowly-growing mycobacteria, including MAC.[38,39] Our results showed a synergistic effect between isepamicin and clofazimine against all of the selected *M. intracellulare* isolates, but in only half of the selected *M. avium* isolates. Thus, isepamicin combined with clofazimine may serve as a promising therapeutic option for the treatment of cavitary, advanced or previously treated *M. intracellulare*-associated diseases. However, more studies are needed to determine the efficacy of this two-drug combination for *M. avium*-associated diseases.

This study has several important limitations. First, the interpretations of the MICs of the eight tested antimicrobials are tentative, and should only serve as predictors of clinical outcomes until clearly confirmed by clinical trials. To date, the efficacy of the tested antimicrobials, except for amikacin and streptomycin, against MAC has only been demonstrated in a few clinical trials.[17,20,23,29,40] Second, the *in vitro* activity of antimicrobials against MAC is currently of questionable clinical significance, and routine susceptibility testing of MAC isolates is recommended for clarithromycin only.[1,41] Third, we have no clinical epidemiological and demographic data to correlate *in vitro* activity. However, as new active drugs become available against MAC, the determination of *in vitro* activity and synergistic activity may provide means to develop better treatment regimens utilizing the most active combinations of existing drugs, although the relevance of our results should be examined *in vivo* such as in animal experiments or clinical studies.

In conclusion, species identification plays an important role when designing treatment regimens for MAC diseases. Isepamicin is as effective as amikacin and streptomycin

against both *M. intracellulare* and *M. avium* isolates and may serve as an alternative aminoglycoside agent if toxicity or adverse effects are major concerns. Both kanamycin and capreomycin are effective for treating *M. intracellulare*-associated diseases but not *M. avium*-associated diseases. D-cycloserine is suitable to treat both *M. intracellulare*- and *M. avium*-associated diseases. Dapsone is not a suitable option alone for the treatment of either *M. intracellulare* or *M. avium*-associated diseases. Clofazimine, either alone or in combination with isepamicin, may be promising for the treatment of *M. intracellulare*-associated diseases but not *M. avium*-associated diseases. However, further clinical trials are needed to confirm our findings.

Conflicts of interest statement

None to declare.

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