



Original article

Efficacy of Mastoparan-AF alone and in combination with clinically used antibiotics on nosocomial multidrug-resistant *Acinetobacter baumannii*Chun-Hsien Lin^a, Mong-Chuan Lee^b, Jason T.C. Tzen^c, Hsien-Ming Lee^d, Sam-Min Chang^e, Wu-Chun Tu^{a,*}, Chuen-Fu Lin^{f,*}^a Department of Entomology, National Chung Hsing University, Taichung, Taiwan^b Graduate Institute of Biotechnology and Biomedical Engineering, Central Taiwan University of Science and Technology, Taichung, Taiwan^c Graduate Institute of Biotechnology, National Chung Hsing University, Taichung, Taiwan^d Department of Nursing, Central Taiwan University of Science and Technology, Taichung, Taiwan^e Department of Medical Laboratory Science and Biotechnology, Feng Yuan Hospital, Ministry of Health and Welfare, Taichung, Taiwan^f Department of Veterinary Medicine, National Chiayi University, Chiayi City, Taiwan

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ABSTRACT

Emergence of multidrug-resistant *Acinetobacter baumannii* (MDRAB) has become a critical clinical problem worldwide and limited therapeutic options for infectious diseases caused by MDRAB. Therefore, there is an urgent need for the development of new antimicrobial agents or alternative therapy to combat MDRAB infection. The aim of this study was to investigate effects of Mastoparan-AF (MP-AF), an amphipathic peptide isolated from the hornet venom of *Vespa affinis* with broad-spectrum antimicrobial activity, on MDRAB. As compared with clinical used antibiotics, MP-AF exhibited potent antimicrobial activity at 2–16 µg/ml against the reference strain *A. baumannii* ATCC 15151 and seven MDRAB clinical isolates, especially the colistin-resistant MDRAB, E0158. The synergistic antimicrobial combination study revealed that MP-AF acted synergistically with specific antibiotics, e.g., ciprofloxacin, trimethoprim/sulfamethoxazole (SXT) or colistin against some isolates of the MDRAB. It was noteworthy when MP-AF combined with SXT exhibited synergistic activity against all SXT-resistant MDRAB isolates. The synergistic combination of MP-AF and antibiotics could reduce the dosage recommended of each antimicrobial agent and improve the safety of medications with ignorable adverse effects, such as colistin with nephrotoxicity in therapeutic dose. Furthermore, MP-AF combined with antibiotics with different antimicrobial mechanisms could reduce selective pressure of antibiotics on bacteria and prevent the emergence of antimicrobial-resistant strains. Importantly, we are the first finding that MP-AF could make MDRAB from the original non-susceptibility to SXT become sensitivity. In conclusion, MP-AF alone or in combination with other antibiotics, especially SXT, is a potential candidate against MDRAB infection in clinical medicine.

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1. Introduction

Acinetobacter baumannii, an aerobic Gram-negative coccobacillus, is an opportunistic pathogen, especially to immunocompromised patients hospitalized in intensive care units (ICUs)

(Bergogne-Bérézín and Towner, 1996). In recent decades, *A. baumannii* has emerged as a common nosocomial pathogen causing severe infections including septicemia, pneumonia, meningitis, urinary tract infections, and infections stemming from wounds (Bergogne-Bérézín and Towner, 1996; Joly-Guillou, 2005; Maragakis and Perl, 2008). Presence of risk factors such as exposure to antimicrobial agents, colonization pressure, or acquisition of genetic elements carrying antibiotic-resistance genes, increase the emerging of multidrug-resistant *A. baumannii* (MDRAB) and has posed a great therapeutic challenge for clinicians (Maragakis and Perl, 2008; Perez et al., 2007). Alternative therapies such as antimicrobial combination therapy or new antimicrobial agents from natural resources, e.g., insect (Hirai et al., 1979), amphibian (Park et al., 1998), and mammalian (Lee et al., 1989), have been

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demonstrated as potential options for combating this microorganism.

Recently, Vila-Farres et al. (2012) reported that antimicrobial peptides from natural resources, particularly mastoparan, effectively restraint the growth of colistin-resistant *A. baumannii*. Mastoparans (MPs), the most abundant peptide from *Vespidae* venoms (Palma, 2006), consist of 14 amino acid residues including several hydrophobic amino acids such as leucine, isoleucine, valine or alanine, 2–4 lysine residues, and an amidated C-terminal residue (Lin et al., 2011; Nakajima, 1984). MPs could form amphipathic helical structures under an appropriate environment, e.g., aqueous trifluoroethanol solution (Chuang et al., 1996; Lin et al., 2011; Yu et al., 2000). MPs exhibit a variety of biological activities, including mast cell degranulation and release of histamine (Chen et al., 2008; Lin et al., 2011), hemolytic activity (Chen et al., 2008; Lin et al., 2011), and activation of phospholipase A₂ and phospholipase C (Rivers et al., 2002). Furthermore, MPs show broad-spectrum antimicrobial activity against both Gram-positive and -negative bacteria (Chen et al., 2008; Ho and Hwang, 1991; Li et al., 2000; Lin et al., 2011), and cause membrane permeabilization on *Escherichia coli* BL21 (Lin et al., 2011).

In our previous study, Mastoparan-AF (MP-AF), isolated from the hornet venom of *Vespa affinis*, presented potent antimicrobial activity, especially against multidrug-resistant *E. coli* isolates from animals (Lin et al., 2012). More importantly, MP-AF displayed ignorable human erythrocyte hemolysis at 32 µg/ml that over the effective antimicrobial concentration to *Citrobacter koseri*, *E. coli*, *Salmonella enterica* Serotype Choleraesuis, and *Vibrio parahaemolyticus* (Lin et al., 2011). In addition, MP-AF also in combination with cephalothin or gentamicin displayed a synergistic activity against *E. coli* clinical isolates (Lin et al., 2012). Therefore, use of MP-AF along with antibiotics harboring different mechanisms of action, could be a feasible strategy to combat MDRAB.

The purpose of this study was to investigate the *in vitro* activity of MP-AF alone or in combination with eight clinically used antibiotics against MDR nosocomial isolates of *A. baumannii* and to evaluate whether this strategy could be as novel therapeutic options for MDRAB infections.

2. Materials and methods

2.1. Bacterial isolates

Seven *A. baumannii* clinical strains were isolated from patients' blood and urine in Laboratory Medicine, Department of Health, Executive Yuan, Fong-Yuan Hospital, Taiwan. In addition, *A. bau-*

mannii ATCC 15151, a reference strain from Bioresource Collection and Research Center (BCRC), Taiwan, was used as a control. Identification of all clinical isolates were further confirmed by PCR and DNA sequencing using bacterium-specific universal primers, 1512F (5'-GTCGTAACAAGGTAGCCGTA-3') and 6R (5'-GGGTYCCC CRTTCRGAAT-3', where Y is C or T and R is A or G) (Chang et al., 2005).

2.2. Genetic analysis of RND efflux pump, OXA-carbapenemase, class 1 integron genes and IS elements

Detection of resistance-nodulation-cell division (RND) efflux pump genes (*adeA*, *adeB*, *adeC*, *adel*, *adeJ*, *adeR* and *adeS*) was performed by PCR using specific primers. The OXA-carbapenemase genes encoding carbapenem-hydrolysing β-lactamases (*bla*_{OXA-23-like}, *bla*_{OXA-51-like} and *bla*_{OXA-58-like}) were analyzed using PCR. In addition, class 1 integron gene (integrase gene *int1*) and IS elements (*ISAb1*, *ISAb2* and *ISAb3*) were also examined by PCR. Sequences of primers used for detecting of antibiotic resistance genes and PCR conditions were shown in Table 1.

2.3. Antimicrobial agents

Mastoparan-AF (MP-AF), INLKAI AALAKKLF-NH₂, was synthesized and purified by Bio Basic Inc. (Ontario, Canada). The crude synthetic MP-AF was further purified by reverse phase HPLC equipped with Sino Chrom ODS-BP (4.6 × 250 mm, 5 µm) under a linear gradient from 35% to 60% (v/v) acetonitrile in water containing 0.1% trifluoroacetic acid, at a flow rate of 1.0 ml/min during 30 min by monitoring at 220 nm. The purity of synthetic MP-AF was over 95%. The molecular weight of the synthetic MP-AF was subsequently confirmed by an electrospray ionization mass spectrometry (ESI-MS). The MP-AF stock solution (10 mg/ml) was prepared by dissolving lyophilized powder in sterile distilled water and stored at –20 °C before use.

Antibiotics tested were purchased from the manufacturers as follows: ampicillin (GERBU, Wieblingen, Germany), cephalothin (Sigma, St. Louis, USA), ciprofloxacin (Fluka, St. Louis, USA), colistin sulfate salt (Sigma, St. Louis, USA), gentamicin (Sigma, St. Louis, USA), neomycin (Sigma, St. Louis, USA), trimethoprim/sulfamethoxazole (SXT) (Sigma, St. Louis, USA), tetracycline (GERBU, Wieblingen, Germany) and tigecycline (Glentham Life Science, Wiltshire, UK). All antibiotics stock solutions (10 mg/ml) were also prepared by dissolving lyophilized powder in distilled water, then sterilized by passing through 0.22 µm filter. All of these sterilized stock solutions were stored at –20 °C until use.

Table 1
Primers for amplify of antibiotic resistant genes and annealing temperatures used in this study.

Target gene	Forward primer sequences (5' → 3')	Reverse primer sequences (5' → 3')	Amplicon size (bp)	Annealing temp (°C)	References
<i>adeA</i>	adeA-F: ATCTTCTGCACGTGTACAT	adeA-R: GGCGTTCACTACTCACTAACC	513	50	Lin et al. (2009)
<i>adeB</i>	adeB-F: TTAACGATAGCGTTGTAACC	adeB-R: TGAGCAGACAATGGAATAGT	541	50	Lin et al. (2009)
<i>adeC</i>	adeC-F: AGCCTGCAATTACATCTCAT	adeC-R: TGGCACTTCACTATCAATAC	560	50	Lin et al. (2009)
<i>adel</i>	adel-F: ATCGCGCTTGTGGTTGTAG	adel-R: AAGCACCAGCCGTTACTGAA	541	50	Lin et al. (2009)
<i>adeJ</i>	adeJ-F: ATTGCACCACCAACCGTAAC	adeJ-R: TAGCTGGATCAAGCCAGATA	453	50	Lin et al. (2009)
<i>adeR</i>	adeR-F: ACTACGATATTGGCGACATT	adeR-R: CCGTCAGATTAAGCAAGATT	447	50	Lin et al., 2009
<i>adeS</i>	adeS-F: TTGGTTAGCCACTGTTATCT	adeS-R: AGTGGACGTTAGGTCAAGTT	544	50	Lin et al. (2009)
<i>int1</i>	Int1F: CAGTGGACATAAGCCTGTTC	Int1R: CCCGAGGCATAGACTGTA	160	50	Koelman et al. (2001)
<i>bla</i> _{OXA23}	OXA-23-like-F: GATCGGATTGGAGAACCAGA	OXA-23-like-R: ATTTCTGACCCAITTTCCAT	501	50	Woodford et al. (2006)
<i>bla</i> _{OXA51}	OXA-51-like-F: TAATGCTTTGATCGGCCTTG	OXA-51-like-R: TGGATTGCACCTTCATCTTGG	353	50	Woodford et al. (2006)
<i>bla</i> _{OXA58}	OXA-58-like-F: AAGTATTGGGCTTGTGCTG	OXA-58-like-R: CCCCTCGCGCTCTACATAC	599	50	Woodford et al. (2006)
<i>ISAb1</i>	ISAb1A: GTGCTTTGGCGCTCATCATGC	ISAb1B: CATGTAACCAATGCTCACC	400	50	Poirel and Nordmann (2006)
<i>ISAb2</i>	ISAb2A: AATCCGAGATAGACGGGTTTC	ISAb2B: TGACACATAACCTAGTGACAC	1,306	52	Poirel and Nordmann (2006)
<i>ISAb3</i>	ISAb3A: CAATCAAATGTCCAACCTGC	ISAb3B: CGTTTACCCCAACATAAGC	374	52	Poirel and Nordmann (2006)

2.4. Antimicrobial susceptibility testing

The antimicrobial disk diffusion susceptibility test was performed by using amikacin (AN, 30 µg), ceftazidime (CAZ, 30 µg), ciprofloxacin (CIP, 5 µg), ceftriaxone (CRO, 30 µg), cefotaxime (CTX, 30 µg), cefepime (FEP, 30 µg), gentamicin (GM, 10 µg), imipenem (IPM, 10 µg), levofloxacin (LVX, 5 µg), minocycline (MI, 30 µg), ampicillin/sulbactam (SAM, 10/10 µg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg) and piperacillin/tazobactam (TZP, 100/10 µg) disks, and the results were interpreted as “susceptible”, “intermediate” or “resistant” according to the Clinical and Laboratory Standards Institute (CLSI) guidelines described in CLSI M100-S22 (CLSI, 2012a).

Minimal inhibitory concentrations (MICs) of antimicrobial agents against *A. baumannii* isolates were determined by the broth micro-dilution method described in CLSI M07-A9 (CLSI, 2012b) with slight modifications. Serial doubling dilutions of antimicrobial agents were made in Mueller Hinton broth (MHB) to the final volume of 100 µl in each well of a 96-well round bottom microtitre plate. Subsequently, 100 µl of bacterial suspension grown in MHB were inoculated into each well to obtain the final concentration of 5×10^5 CFU/ml and then incubated at 35 °C for 24 h. After 24 h of incubation, plates were read for visual turbidity and estimated based on optical density at 620 nm in each well. The MIC is defined as the lowest concentration of antimicrobial agent that showing no visual bacterial growth (optically clear well). Experiments were performed in duplicates in three independent experiments. The MICs are presented as the median values obtained in duplicates from three independent experiments.

2.5. Synergistic antimicrobial combination studies

In combination studies, *A. baumannii* clinical isolates were used to test the antimicrobial combinations by a chequerboard titration method using 96-well round bottom microtitre plate. MP-AF was diluted by serial 2-fold steps vertically and antibiotic was also diluted in 2-fold steps horizontally. The ranges of MP-AF dilutions used were from 64 to 0.5 µg/ml, concentrations of colistin were from 32 to 0.0625 µg/ml, and those for seven antibiotics were from 256 to 0.5 µg/ml. The fractional inhibitory concentration (FIC) index for the combination of two antimicrobials was calculated from the following equation: FIC index = $FIC_A + FIC_B = A/MIC_A + B/MIC_B$, where A and B are the MICs of drug A and drug B in the combination, MIC_A and MIC_B are median MICs of drug A and drug B alone, and FIC_A and FIC_B are FICs of drug A and drug B. The FIC indexes were interpreted as follows: $FIC \leq 0.5$, synergy; $0.5 < FIC < 1$, partial synergy; $FIC = 1$, additivity; $1 < FIC < 4$, indifference; $FIC \geq 4.0$, antagonism (Dawis et al., 2003). The FIC indexes were obtained from three independent experiments.

3. Results

3.1. Analysis of RND efflux pump, OXA-carbapenemase, *int1* genes and IS elements in *A. baumannii* clinical isolates

Genetic analysis of seven *A. baumannii* isolates, shown in Table 2, revealed that all *A. baumannii* clinical isolates were PCR positive for RND efflux pump and *int1* genes. As Krahn et al. (2015) described, *A. baumannii* ATCC 15151 was also positive for RND efflux pump. OXA-carbapenemase was associated with *bla*_{OXA23}, *bla*_{OXA51} and *bla*_{OXA58} genes. Among these genes detected, *bla*_{OXA23} and *bla*_{OXA51} was detected in all *A. baumannii* isolates, but *bla*_{OXA58} was not detected. ISAb1 was detected in all *A. baumannii* isolates, but none of ISAb2 and ISAb3 was detected in tested isolates.

The 16S-23S rRNA gene spacer region sequences of 7 MDRAB were submitted to GenBank. Accession numbers were shown as follows: E0158 (KR732279), E0407 (KR732281), E0469 (KR732282), E0528 (KR732283), E0682 (KR732284), E0948 (KR732286) and E1359 (KR732287).

3.2. In vitro activity of MP-AF and clinically used antibiotics against *A. baumannii* clinical isolates

Antimicrobial susceptibility profiles of the seven *A. baumannii* clinical isolates tested by different types of antibiotics were shown in Table 3. It is found that all isolates were susceptible to MI, but resistant to GM, TZP, CAZ, CTX, CRO, IMP, CIP and SXT, and most of them were resistant to AN (5/7), SAM (4/7), FEP (6/7), LVX (5/7). Interpretive criteria for tigecycline MICs were based on the United States Food and Drug Administration (FDA) criteria (Altun et al., 2014; Navon-Venezia et al., 2007), it showed that all isolates were also susceptible to tigecycline. According to MIC interpretive criteria described by CLSI (2012a), it revealed that six isolates were susceptible to colistin except E0158. As mentioned above, all clinical isolates were resistant to at least one antimicrobial agent in three or more antimicrobial classes and could be defined as MDRAB based on definition for multi-drug resistant (MDR) bacteria described by Magiorakos et al. (2012). In addition, *A. baumannii* ATCC 15151 was susceptible to antibiotics tested and it also corresponds with Bonnin et al. (2012) described.

The median MICs of MP-AF and nine antibiotics tested against the reference strain, ATCC 15151, and seven MDRAB isolates are summarized in Table 4. MP-AF exhibited potent antimicrobial activity against *A. baumannii* tested, at 2 µg/ml against ATCC 15151, 4 µg/ml against E0469, E0528, E0682 and E0948, 8 µg/ml against E0158 and E0407 and 16 µg/ml against E1359. Colistin showed low MICs, at 1 µg/ml for E0407, E0948, E1359 and ATCC

Table 2

Resistance-nodulation-cell division efflux pump genes, class 1 integron gene, OXA-carbapenemase genes and IS elements detected in *A. baumannii* clinical isolates.

<i>A. baumannii</i> isolates	Chromosome								Plasmid						
	Resistance-nodulation-cell division (RND) efflux pump genes								Class 1 integron gene	OXA-carbapenemase genes			IS elements		
	<i>adeA</i>	<i>adeB</i>	<i>adeC</i>	<i>adel</i>	<i>adeJ</i>	<i>adeR</i>	<i>adeS</i>	<i>int1</i>		<i>bla</i> _{OXA23}	<i>bla</i> _{OXA51}	<i>bla</i> _{OXA58}	ISAb1	ISAb2	ISAb3
E0158	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
E0407	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
E0469	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
E0528	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
E0682	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
E0948	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
E1359	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
ATCC 15151	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–

+, detected; –, not detected.

Table 3
Antimicrobial susceptibility profiles of *A. baumannii* clinical isolates in this study.

A. baumannii isolates	Year	Origins	Antimicrobial susceptibility profiles for <i>A. baumannii</i> clinical isolates													
			Antimicrobial classes													
			Aminoglycoside		β-Lactam/β-lactamase inhibitor combination		Cephem			Penem	Quinolone		Folate pathway inhibitor	Tetracycline		
AN	GM	SAM	TZP	CAZ	CTX	CRO	FEP	IMP	CIP	LVX	SXT	MI				
E0158	2007	Blood	R	R	S	R	R	R	R	R	I	R	R	I	R	S
E0407	2008	Urine	R	R	I	R	R	R	R	R	R	R	R	R	R	S
E0469	2008	Urine	S	R	R	R	R	R	R	R	R	R	R	R	S	
E0528	2008	Blood	S	R	I	R	R	R	R	R	R	R	R	R	S	
E0682	2008	Urine	R	R	R	R	R	R	R	R	R	R	R	R	S	
E0948	2009	Blood	R	R	R	R	R	R	R	R	R	R	R	R	S	
E1359	2010	Urine	R	R	R	R	R	–	R	R	R	R	–	R	S	
ATCC 15151			S	S	S	S	S	S	S	S	S	S	S	S	S	

–, not detected.

Abbreviations: AN, amikacin; GM, gentamicin; SAM, ampicillin/sulbactam; TZP, piperacillin/tazobactam; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP, cefepime; IMP, imipenem; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole; MI, minocycline; S, susceptible; I, intermediate; R, resistant.

15151, 2 µg/ml for E0469, E0528 and E0682, and 4 µg/ml for E0158. Ampicillin, cephalothin and gentamicin could not inhibit growth of isolates at 256 µg/ml, but showed low MICs of 16, 8 and 1 µg/ml against ATCC 15151. Ciprofloxacin presented MICs of 64 and 0.5 µg/ml against E0407 and ATCC 15151, respectively, but has no effects on other bacterial growth even at the concentration of 256 µg/ml. Neomycin displayed MICs at 1 µg/ml for ATCC 15151, 256 µg/ml for E0469 and E0948, but no inhibitory effects on the rest of isolates at 256 µg/ml. SXT showed MICs at 64 µg/ml for E0682, 256 µg/ml for E0407 and 2 µg/ml for ATCC 15151, but no activity against other isolates at 256 µg/ml. Tetracycline exhibited activity against *A. baumannii* at 4 µg/ml for ATCC 15151, 128 µg/ml for E0407, E0469 and E0948, and 256 µg/ml for E0528 and E0682, but showed ineffective against E0158 and E1359 even at 256 µg/ml. Tigecycline showed the lowest MICs, at 0.0625 µg/ml for E0528, 0.125 µg/ml for E0682, E0948 and ATCC 15151, 0.25 µg/ml for E0407 and E0469 and 0.5 µg/ml for E0158 and E1359. Except colistin and tigecycline, MP-AF presented superior antimicrobial activity against *A. baumannii* clinical isolates than other antibiotics used in this study.

3.3. Evaluation of the efficacy of MP-AF combined with clinically used antibiotics against MDRAB

The efficacy of MP-AF combined with clinically used antibiotics against MDRAB was estimated by FIC indexes as shown in Table 5. MP-AF combined with colistin presented synergistic activity

against E0158, E0407, E0469, E0528, E0948 and E1359, showing FIC indexes between 0.125 and 0.375, and revealed indifferent effect on E0682. When MP-AF combined with SXT, it also exhibited synergistic activity against E0158, E0407, E0469, E0528, E0948 and E1359, showing FIC indexes between 0.25 and 0.5, and showed partial synergy against E0682. As MP-AF plus ciprofloxacin, it presented synergistic activity against E0528 and E0948, showing same FIC index of 0.375. When MP-AF combined with tetracycline, it displayed partial synergy against E0407, showing FIC index of 0.531, and additivity against E0469, E0528, E0682 and E1359, showing same FIC index of 1. However, MP-AF showed indifferent effects on all MDRAB when combined with ampicillin, cephalothin, gentamicin and neomycin with FIC indexes between 1.002 and 2.004.

4. Discussion

Gram-negative bacteria can resist the action of antibiotics by several mechanisms, e.g., the resistance-nodulation-cell division (RND) efflux pump in *A. baumannii*. In this study, RND efflux pump genes *adeABC*, *adeIJK*, and *adeSR* were found in all *A. baumannii* clinical isolates. The AdeABC efflux pump, encoded by *adeABC* genes, could pump a broad spectrum of antibiotics, e.g., aminoglycosides, cefotaxime, tetracyclines, erythromycin, chloramphenicol, trimethoprim, or fluoroquinolones to reduce antibiotics intake (Fournier et al., 2006; Magnet et al., 2001). Similar to AdeABC, AdeIJK also offers resistance to β-lactams, chloramphenicol, tetracyclines, erythromycin, fluoroquinolones, fusidic acid, novobiocin,

Table 4
Median minimal inhibitory concentration of MP-AF and clinical used antibiotics against *A. baumannii* clinical isolates.

A. baumannii isolates	Median MIC (µg/ml)									
	MP-AF	AMP	CF	CIP	CL	GM	NEO	SXT	TET	TGC
E0158	8	>256	>256	>256	4	>256	>256	>256	>256	0.5
E0407	8	>256	>256	64	1	>256	>256	256	128	0.25
E0469	4	>256	>256	>256	2	>256	256	>256	128	0.25
E0528	4	>256	>256	>256	2	>256	>256	>256	256	0.0625
E0682	4	>256	>256	>256	2	>256	>256	64	256	0.125
E0948	4	>256	>256	>256	1	>256	256	>256	128	0.125
E1359	16	>256	>256	>256	1	>256	>256	>256	>256	0.5
ATCC 15151	2	16	8	0.5	1	1	1	2	4	0.125

Abbreviations: MIC, minimal inhibitory concentration; MP-AF, mastoparan-AF; AMP, ampicillin; CF, cephalothin; CIP, ciprofloxacin; CL, colistin; GM, gentamicin; NEO, neomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TGC, tigecycline.

Table 5FIC indexes of combination studies between MP-AF and clinical used antibiotics against *A. baumannii* clinical isolates.

Antibiotics	FIC index (Conc. _{MP-AF} /Conc. _{antibiotic}), MP-AF						
	E0158	E0407	E0469	E0528	E0682	E0948	E1359
Ampicillin	1.008(8/2)	1.004(8/0.5)	1.008(4/2)	1.002(4/0.5)	1.004(4/1)	1.002(4/0.5)	1.002(16/0.5)
Cephalothin	1.004(8/1)	1.008(8/2)	1.004(4/1)	1.004(4/1)	1.031(4/8)	1.004(4/1)	1.002(16/0.5)
Ciprofloxacin	1.125(8/32)	1.016(8/1)	1.002(4/0.5)	0.375(0.5/64)	1.016(4/4)	0.375(0.5/64)	1.002(16/0.5)
Colistin	0.188(1/0.25)	0.313(0.5/0.25)	0.25(0.5/0.25)	0.25(0.5/0.25)	2(4/2)	0.375(1/0.125)	0.125(1/0.0625)
Gentamicin	2.002(16/0.5)	2.002(16/0.5)	2.004(8/1)	2.002(8/0.5)	1.002(4/0.5)	2.002(8/0.5)	2.002(32/0.5)
Neomycin	2.002(16/0.5)	2.002(16/0.5)	2.002(8/0.5)	2.004(8/1)	2.002(8/0.5)	2.002(8/0.5)	1.004(16/1)
SXT	0.5(2/64)	0.5(2/64)	0.25(0.5/32)	0.25(0.5/32)	0.625(0.5/32)	0.375(0.5/64)	0.5(4/64)
Tetracycline	1.008(8/2)	0.531(4/4)	1(2/64)	1(2/128)	1(2/128)	1.004(4/0.5)	1(8/128)

Abbreviations: MP-AF, mastoparan-AF; SXT, trimethoprim/sulfamethoxazole.

The concentrations of MP-AF dilutions used were from 64 to 0.5 µg/ml, colistin were from 32 to 0.0625 µg/ml and those for seven antibiotics were from 256 to 0.5 µg/ml. The FIC indexes were defined as follows: FIC ≤ 0.5, synergy (bolded); 0.5 < FIC < 1, partial synergy; FIC = 1, additivity; 1 < FIC < 4, indifference; FIC ≥ 4.0, antagonism (Dawis et al., 2003).

and trimethoprim (Damier-Piolle et al., 2008). As mentioned above, it could interpret that why *A. baumannii* isolates were resistant to antibiotics tested as shown in Table 4. The *adeSR* genes, encoding sensor kinase and regulating the two-component system, were adjacent to *adeABC* genes, transcribed in the opposite direction, and located upstream from *adeA* (Marchand et al., 2004; Wiczorek et al., 2008). The AdeSR two-component system could control the expression of AdeABC efflux pump by monitoring the environmental conditions (Marchand et al., 2004).

Our study demonstrated that MP-AF exhibited potent antimicrobial activity against the MDRAB and the reference strain, *A. baumannii* ATCC 15151, as compared with clinically used antibiotics. Vila-Farres et al. (2012) had evaluated the activity of 15 antimicrobial peptides against colistin-susceptible and colistin-resistant *A. baumannii*. It was noteworthy that mastoparan, isolated from the venom of *Vespa lewisii* (Hirai et al., 1979), showed better antimicrobial activity with lower MICs against colistin-susceptible and colistin-resistant *A. baumannii* than other antimicrobial peptides, e.g., buforin I, histatin 5, magainin II and β-defensin, from other natural resources (Vila-Farres et al., 2012).

MP-AF also showed effective antimicrobial activity with MICs ranging from 2 to 16 µg/ml against MDRAB isolates. Our previous study verified that MP-AF exhibited a broad-spectrum antimicrobial activity against both Gram-positive and -negative bacteria and displayed rapid bacterial killing activity against multidrug-resistant *E. coli* (Lin et al., 2012, 2011). In addition, the cytotoxicity of MP-AF also has been evaluated by hemolytic activity assay and mast cell degranulation assay. MP-AF displayed negligible hemolytic activity on human erythrocytes, approximately 0.59% hemolysis, at the concentration of 32 µg/ml, and showed slight mast cell degranulation, 9.49%, at the concentration of 16 µg/ml (Lin et al., 2011). It suggested that MP-AF could be non-toxic or displayed minor adverse effects on human body when exhibiting antimicrobial activity against MDRAB at effective dosages ranging from 2 to 16 µg/ml. Furthermore, when MP-AF interacted synergistically with antibiotics, it meant that the 4-fold or greater decrease in MIC of both antimicrobial agents in combination compared with antimicrobial agents tested individually. Even these combinations showed additive activity against MDRAB, it meant that the 2-fold decrease in MIC with both antimicrobial agents. Therefore, MP-AF combined with antibiotics could lower MP-AF dosage, reduce or abolish its mild adverse effects and improve its safety. It believed that MP-AF combined with antibiotics could be potential as a therapeutic alternative to combat MDRAB.

According to the results of analysis of RND efflux pump genes in MDRAB and *in vitro* activity of MP-AF against MRDAB, it found that RND efflux pumps had no effects on the antimicrobial efficacy of MP-AF against MDRAB isolates. MP-AF targeted membrane and caused severe permeabilization on *E. coli* BL21 at low concentra-

tion (Lin et al., 2011). In addition, scanning electron microscopy (SEM) observation also verified that MP-AF acted on the surface of *E. coli* and caused irregular dents and perforations (Shyu et al., unpublished data) similar to mastoparan-M described by Li et al. (2000). As mentioned above, it suggested that antimicrobial potency of MP-AF could be unaffected by mechanism of antibiotic resistance in MDRAB. Thus, MP-AF has potential as alternative antimicrobial agent for humans against MDRAB infection in the clinical application.

Owing to lack of new antimicrobial agents and limited therapeutic options for *A. baumannii* infection, new uses for old drugs, e.g., first-line antibiotic, SXT and highly adverse drug, colistin, has been proposed as a potential strategy to combat *A. baumannii* infection. However, the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program revealed that *A. baumannii* displayed higher resistance rate to SXT exceeded 70% over the past ten years, 2000–2010 (Kuo et al., 2012; Lauderdale et al., 2004). In the antimicrobial susceptibility testing, MDRAB isolates were almost resistant to these antibiotics tested, including ciprofloxacin and SXT. However, when ciprofloxacin or SXT combined with MP-AF, it exhibited synergistic activity against some MDRAB isolates. Interestingly, it also found that SXT combined with MP-AF was particularly effective against MDRAB isolates, being resistant to SXT. It suggested that with the aid of MP-AF, MDRAB isolates could be susceptible to SXT.

Mechanisms of antimicrobial resistance mainly included enzymatic degradation of antibiotics, alteration of antibiotic target proteins and decreased membrane permeability to antibiotics. SXT and ciprofloxacin resistance could be resulted from decreased membrane permeability. MP-AF has been verified that it could cause bacterial membrane permeabilization drastically at low concentration (Lin et al., 2011). Thus, it suggested that MP-AF, the membrane-active peptide, could play an important role in synergistic combination against MDRAB. Our study speculated that MP-AF causes membrane permeabilization to allow ciprofloxacin or SXT, which act intracellularly, to facilitate cellular uptake and then against the bacteria. This process is similar to how other antimicrobial peptides acted synergistically with clinically used antibiotics (Giacometti et al., 2005a,b,c; Lin et al., 2012).

Colistin, used as a comparator, also showed potent activity against MDRAB isolates. In Taiwan, colistin is not a common treatment option for *A. baumannii*-infected patients and has been out of stock since 2009. Due to the adverse effect of colistin, nephrotoxicity, it could be explained why most *A. baumannii* isolates were susceptible to colistin. Owing to nephrotoxicity, treatment of *A. baumannii* infection by colistin should be recommended by medical advice. MP-AF in combination with colistin presented potent synergistic activity against MDRAB isolates. In addition, MP-AF/colistin combination could reduce at least 4-fold dosages of MP-

AF and colistin; hence, it decreased adverse effects of colistin and also increased its safety in treating *A. baumannii*-infected patients. Previous clinical experience revealed that excessive and inappropriate use of antibiotics could accelerate emergence of antibiotic-resistant clinical isolates. However, we pointed out the feasible strategy, compound medication composed of naturally derived cationic antimicrobial peptide and chemical compound. These synergistic combinations could lower effective antimicrobial dosage to minimize adverse effect of antibiotics. Owing to decrease of antimicrobial dosage, it could reduce the selective pressure on pathogens to retard the emergence of antibiotic resistance. Furthermore, it also could elongate lifespan of antibiotics and gain more time to develop the new class of antibiotics.

Bacteria containing antimicrobial resistance genes affected the efficiency of antimicrobial combination therapy (Lin et al., 2012). To overcome this problem, it is crucial to develop accurate and rapid diagnosis based on genetic analyses to detect antimicrobial resistance genes in pathogenic bacteria. Precise diagnosis will help clinicians to choose the appropriate antimicrobial combination therapy to treat specific bacterial infections.

This study revealed that MP-AF produced better antimicrobial results against MDRAB than antibiotics. MP-AF also acted synergistically with certain antibiotics, such as ciprofloxacin, SXT or colistin. Synergistic antimicrobial combination exhibited greater antibacterial activity than that would be expected from each drug individually. These results show that MP-AF alone or combined with other antibiotics has the potential to develop as a promising candidate for *A. baumannii* infections.

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