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Antibacterial activity of the recombinant antimicrobial peptide Ib-AMP4 from *Impatiens balsamina* and its synergy with other antimicrobial agents against drug resistant bacteria

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Dedicated to Prof. Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

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Ib-AMP4 is an antimicrobial peptide of *Impatiens balsamina* (Balsaminaceae). Ib-AMP4 was produced as a recombinant peptide and in this study its antimicrobial activity against human bacterial pathogens was investigated. Ib-AMP4 was bactericidal against both Gram positive and Gram negative bacteria with MIC values between 0.49 and 3.5 μM in sensitive species. A genuine synergistic effect was achieved when IB-AMP4 was employed in combination with the plant monoterpene thymol against drug-resistant *Klebsiella pneumoniae* (KPC) ATCC700603, or with the antibiotics vancomycin or oxacillin against *Enterococcus faecalis* (VRE) ATCC51299.

1. Introduction

As a consequence of the increased abuse of antibiotics in medicine and agriculture, drug resistance has widely spread all over the world. A surveillance taken in 2009 reported that more than 15% of clinical *E. coli* isolates were resistant to the third generation of cephalosporin (World-Health-Organization 2011). In addition, in Latin America, MRSA is common and more than 26% of clinical isolates are drug resistant (World-Health-Organization 2007). Along with the increase of drug resistance, research and development for new antibiotics have slowed down. Only five new antimicrobial agents were developed between 2003 to 2007 while there had been 16 new antibiotics between 1983 to 1987 (Spellberg et al. 2004). Thus, a severe bottleneck could occur in the future and people face a similar situation as in the pre-antibiotics era when a simple infection could be fatal. Therefore, the development of novel antibiotics with new antimicrobial mechanisms is an urgent need.

Antimicrobial peptides (AMP) are a group of peptides with a broad microbicidal activity against bacteria and fungi (Nguyen et al. 2011). Ib-AMP4, an AMP from *Impatiens balsamina* (Balsaminaceae) can selectively target the bacterial plasma membrane, forming pore channels, which disable cellular respiration and make cells leaky so that metabolites and ions can diffuse out of the cells (Nguyen et al. 2011). Furthermore, it has been proposed that Ib-AMP4 could be safe when used *in vivo* against infections since it showed neither a haemolysis of erythrocytes nor a perturbation of the biomembrane of fibroblasts (Tailor et al. 1997).

2. Investigations, results and discussion

In order to have sufficient amounts of Ib-AMP4, the peptide was produced in our laboratory by recombinant DNA technique (Fan et al., in preparation). The antimicrobial efficacy of Ib-AMP4 against human bacterial pathogens was determined by recording the minimal inhibition concentration (MIC) accord-

Table 1: MIC values for recombinant Ib-AMP4 against Gram positive and Gram negative bacteria

Species	ATCC No.	MIC (μM)
Gram positive		
Bacillus megaterium	14581	0.49
Bacillus subtilis	6051	0.98
Micrococcus luteus	7468	1.97
Enterococcus casseliflavus	700327	1.57
Enterococcus faecalis	29212	15.74
VRE	51299	31.48
Staphylococcus aureus	29213	3.15
	25923	3.15
MRSA	NCTC10442	9.84
Staphylococcus epidermidis	14990	31.48
Staphylococcus oralis	35037	31.48
Staphyloccus saprophyticus	15305	0.98
Streptococcus agalactiae	27956	62.97
Streptococcus pneumoniae	49619	7.87
Streptococcus pyogenes	12344	3.15
Gram negative		
Klebsiella oxytoca	700324	15.74
Klebsiella pneumoniae (KPC)	700603	47.23
Escherichia coli	25922	3.15
	35150	3.15
Pseudomonas aeruginosa	27853	62.97

MRSA methicillin resistant *Staphylococcus aureus;* VRE vancomycin resistant *Enterococcus;* KPC carbapenemase producing *Klebsiella pneumoniae*

Table 2: Combination of Ib-AMP4 with other antimicrobial agents in resistant pathogens (VRE, KPC): Results of checkerboard analysis

	K. pneumonia ATCC700603			*E. faecalis ATCC51299*	
Combination*	MIC[1]	FIC[2]	Combination*	MIC[1]	FIC[2]
$AgNO_3$ (8.7 μg/ml)			$AgNO_3$ (45 μg/ml)		
1.75 μg/ml + P	41.98	1.09	7.5 μg/ml + P	27.99	1.06
2.63 μg/ml + P	27.99	0.89	15 μg/ml + P	27.99	1.22
3.5 μg/ml + P	12.44	0.66	22.5 μg/ml + P	1.64	0.55
4.38 μg/ml + P	8.29	0.68	36 μg/ml + P	0.48	0.68
Thymol (750 μg/ml)			Thymol (625 μg/ml)		
93.75 μg/ml + P	47.23	1.13	62.5 μg/ml + P	18.66	0.69
187.5 μg/ml + P	12.44	0.51	125 μg/ml + P	12.44	0.59
281.25 μg/ml + P	5.53	*0.49*	186.5 μg/ml + P	12.44	0.70
375 μg/ml + P	5.53	0.62	250 μg/ml + P	5.53	0.58
468.75 μg/ml + P	3.68	0.70	312.5 μg/ml + P	2.46	0.58
562.5 μg/ml + P	2.46	0.80	375 μg/ml + P	1.09	0.63
656.25 μg/ml + P	1.64	0.91	437.5 μg/ml + P	0.48	0.72
EDTA (745 μg/ml)			Vancomycin (7.5 μg/ml)		
93.13 μg/ml + P	18.66	0.52	0.75 μg/ml + P	31.48	1.10
186.25 μg/ml + P	18.66	0.65	1.5 μg/ml + P	18.66	0.79
279.38 μg/ml + P	18.66	0.77	2.25 μg/ml + P	5.53	*0.47*
372.5 μg/ml + P	12.44	0.76	3 μg/ml + P	3.68	0.52
465.63 μg/ml + P	12.44	0.89	3.75 μg/ml + P	1.64	0.55
558.75 μg/ml + P	12.44	1.01	4.5 μg/ml + P	1.64	0.65
651.88 μg/ml + P	12.44	1.14	5.25 μg/ml + P	1.64	0.75
Vancomycin (120 μg/ml)			Oxacillin (32 μg/ml)		
12 μg/ml + P	47.23	1.1	6.4 μg/ml + P	10.07	0.52
24 μg/ml + P	47.23	1.2	9.6 μg/ml + P	7.56	0.54
36 μg/ml + P	47.23	1.3	12.8 μg/ml + P	0.94	*0.43*
48 μg/ml + P	47.23	1.4	16 μg/ml + P	0.94	0.53

* The checkerboard method was used to evaluate the combinations. [1] MIC values (in μM) were determined for Ib-AMP4 in media with serial dilution (ranging from 0.1x MIC to 1x MIC) of the given combination partner. Values in brackets (after antimicrobial agents) represent the MIC values (in μg/ml). P = Ib-AMP [2] FIC = $FIC_A + FIC_B$ (FIC_A = MIC (A in combination with B)/MIC (A alone); FIC_B = MIC (B in combination with A)/MIC (B alone)). FIC > 2.0 indicates antagonistic effects, 0.5 < FIC < 2.0 additive, and FIC < 0.5 synergistic effects (shown in italics).

ing to the NCCLS broth microdilution method (NCCLS 2003). Ib-AMP4 exhibited a broad activity against both Gram positive and Gram negative bacteria (Table 1) with MICs between 0.49 and 3.5 μM in sensitive bacteria. A few bacteria, such as *Enterococcus faecalis, Streptococcus agalactiae, Staphylococcus oralis, Staphylococcus epidermidis, Klebsiella oxytoca*, and *Pseudomonas aeruginosa*, were less sensitive (MIC between 15 and 63 μM). Differences may be due to cell wall and biomembrane composition (de la Fuente-Nunez et al. 2012).

Bactericidal kinetics were determined by counting viable bacteria after treatment with 2 X MIC of Ib-AMP4. The viable colonies were counted after incubation of 24 h. The bactericidal effect was rapid as compared to conventional antibiotics and 99% bacteria were killed within 1 h after the treatment of Ib-AMP4 (data not shown).

In a third set of experiments we tested the potential synergy of combinations of Ib-AMP4 with established antimicrobial agents. Silver has been used as antibacterial agent for centuries before the discovery of the first modern antibiotic, such as penicillin. It could kill bacteria either indirectly by producing highly reactive oxygen species or directly by inactivating proteins (Davies and Etris 1997). The plant monoterpene thymol is another compound with a long history which could even be traced back to as early as ancient Egypt when Egyptians used thymol to preserve mummies. Thymol interacts with bacterial membranes. In addition, being a phenolic compound it can dissociate to a phenolate ion under physiological conditions which can interact with proteins (Trombetta et al. 2005; Wink 2008). EDTA causes instability of the cell membrane by chelating divalent cations. It has already been used in combination with traditional antibiotics to overcome drug resistance (Aoki et al. 2010; Berges et al. 2007; Martin-Visscher et al. 2011; Raad et al. 2007). Vancomycin and oxacillin are cell wall inhibitors blocking the crosslinking of peptidoglycan net. A promising combination of Ib-AMP4 with these antimicrobial agents was expected because their modes of action differed.

Ib-AMP4 was applied in combination with silver nitrate, thymol, EDTA, oxacillin and vancomycin against MDR bacteria, such as *Klebsiella pneumoniae* ATCC700603 (KPC) and *Enterococcus faecalis* ATCC51299 (VRE) (Table 1). About 50 two-drug combinations were tested in total (Table 2). Our data showed that all of the six compounds already inhibited the growth of MDR strains when they were applied individually. However, combinations with Ib-AMP4 were more powerful. These combinations (studied by the checkerboard method) exhibited at least an additive effect, but a genuine synergistic effect could be detected when Ib-AMP4 was used in two-drug combination with thymol against *K. pneumoniae* ATCC700603, or with vancomycin and oxacillin against *E. faecalis* ATCC51299 (Table 2).

In conclusion, Ib-AMP4 is bactericidal against a wide range of Gram positive and Gram negative bacteria. It is bactericidal within a short time period after application as compared to a rather slow activity of conventional antibiotics. Combination of Ib-AMP4 with other antimicrobial agents can be synergistic and even be more powerful. Animal experiments with optimized formulated IB-AMP4 preparations (with and without synergists) are required to prove its efficacy *in vivo.*

3. Experimental

MIC tests were carried out in dextrose tryptone broth with or without additional 2.5% sheep blood. Ib-AMP4 was dissolved in ddH_2O with 5 μg/ml H_2O_2 to a final concentration of 400 μg/ml (to activate its disulfide bridges)

and then stored overnight at 4 °C. The activated peptide was tested against 20 standard ATCC bacteria (Table 1).

For bactericidal kinetics determination, *Staphylococcus aureus* ATCC25923 was seeded and grown in fresh dextrose-tryptone-broth DTB medium overnight. The overnight culture was then transferred into fresh DTB containing 20 μg/ml Ib-AMP4. Every 2 min after incubation 10 μl aliquots of the culture medium were diluted into 990 μl ddH_2O, and 100 μl of the dilution were immediately plated on sheep blood agar plate (37 °C).

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